Universidade do Minho
Escola de Engenharia

Joana Isabel Ferreira Alves

Microbiology of thermophilic anaerobic syngas conversion

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Microbiology of thermophilic anaerobic syngas conversion

Tese de Doutoramento em Engenharia Química e Biológica

Trabalho efectuado sob a orientação da
Doutora Diana Zita Machado de Sousa
da
Doutora Maria Madalena dos Santos Alves
e da
Doutora Caroline M Plugge

Julho de 2013

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Título da tese:
Microbiology of thermophilic anaerobic syngas conversion
Microbiologia da conversão anaeróbia de gás de síntese em condições termofílicas

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Summary

Biological syngas fermentation can be used for novel biotechnological applications. Syngas can be produced from biomass and recalcitrant wastes; fermentation of this gas is therefore an environmentally friendly process with foreseen applications in the production of biofuels and chemicals. This thesis reports the use of thermophilic anaerobic mixed cultures for syngas and carbon monoxide (CO) conversion, as well as the isolation and characterization of two new thermophilic bacterial strains - one CO-utilizing bacterium and one CO-tolerant bacterium.

Stable thermophilic enrichments converting syngas or CO at 55 °C were obtained by long term exposure of a thermophilic anaerobic suspended sludge to these substrates. Enrichments were successively transferred (for over a year) with syngas and pure CO, as sole carbon and energy source. CO partial pressure was increased from 0.09 to 0.88 bar during the enrichment procedure. Enrichment cultures initiated with syngas produced mainly acetate, while hydrogen was the main product detected in enrichment cultures initially incubated with CO. Bacteria branching within the families *Peptococcaceae* and *Thermoanaerobacteraceae* were present in syngas and CO enrichment cultures. Syngas enrichment cultures were composed of two predominant species related to *Desulfotomaculum* and *Caloribacterium* genera, while bacteria assigned to *Thermincola* and *Thermoanaerobacter* genera were abundant in CO enrichment cultures.

The effect of sulfate on CO conversion by the obtained syngas-degrading enriched culture was also investigated. Although the enrichment culture could convert CO into mainly acetate both in the presence and in the absence of sulfate, CO conversion was faster when sulfate was present. Identification of the predominant microorganisms revealed the presence of *Desulfotomaculum*-like bacteria.

A novel CO-tolerating bacterium, *Thermoanaerobacter carboxyditolerans*, was isolated from the syngas-degrading enriched culture. This bacterium is closest related to *Thermoanaerobacter thermohydrosulfuricus* (97% identity based on 16S rRNA gene sequence). Although *T. carboxyditolerans* does not utilize CO, it is able to grow in the presence of high CO concentrations (pCO = 1.7 bar). Other *Thermoanaerobacter* species showed tolerance to CO, namely *T. thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* and *T. wiegelii*; growth of these bacteria was not substantially affected by CO concentrations ranging from 25% to 100% (pCO from 0.425 to 1.7 bar). Nevertheless, hydrogen production by those species decreased with increasing CO partial pressure.

A new thermophilic hydrogenogenic carboxydotrophic bacterium, *Moorella stamsii*, could be isolated from the CO-degrading enrichment. This bacterium is able to utilize CO coupled to the production of hydrogen. *M. stamsii* is phylogenetically related to *Moorella glycerini* (97% identity based on 16S rRNA gene sequences).

With this PhD research we gained insight in the ecophysiology of thermophilic anaerobic conversion of syngas and CO. Results presented in this thesis are important for prospecting biotechnological processes for the production of added-values compounds, such as hydrogen and/or acetate.

Sumário

A fermentação biológica de gás de síntese pode ser usada como um novo processo biotecnológico para produção de biocombustíveis. O gás de síntese pode ser produzido a partir de biomassa florestal ou de resíduos recalcitrantes; a fermentação biológica deste gás é um processo ambiental e economicamente sustentável, com potenciais aplicações na produção de biocombustíveis e outros compostos químicos de elevado interesse. Nesta tese são descritos estudos que utilizam culturas mistas, em condições termofílicas, com o objetivo de estudar a conversão de gás de síntese e de monóxido de carbono (CO) por essas culturas. Descreve-se também o isolamento e caracterização fisiológica de duas novas bactérias; uma das bactérias isoladas é capaz de utilizar o CO e a outra é tolerante a elevadas concentrações de CO.

Culturas estáveis e enriquecidas em gás de síntese ou CO, em condições termofílicas 55 °C foram conseguidas após longa exposição da biomassa suspensa anaeróbia (usada como inóculo) a estes substratos. As culturas foram sucessivamente transferidas para meio novo (durante mais de um ano), com gás de síntese ou CO como única fonte de carbono e energia. Durante o processo de enriquecimento das culturas, a pressão parcial de CO foi aumentando, começou por 0.09 bar até atingir 0.88 bar. As culturas iniciadas com gás de síntese como substrato, produziram maioritariamente acetato, enquanto que as culturas que foram inicialmente incubadas com CO produziram maioritariamente hidrogénio. Verificou-se que a comunidade bacteriana dominante presente nas culturas enriquecidas em gás de síntese e CO pertence às famílias *Peptococcaceae* and *Thermoanaerobacteraceae*. Os enriquecimentos em gás de síntese apresentaram duas espécies dominantes, filogeneticamente relacionadas com os géneros *Desulfotomaculum* e *Caloribacterium*, enquanto que bactérias pertencentes aos géneros *Thermincola* e *Thermoanaerobacter* estavam abundantemente presentes nos enriquecimentos em CO.

O efeito do sulfato na conversão de CO pela cultura enriquecida em gás de síntese anteriormente obtida foi também objeto de estudo. Embora a cultura enriquecida consiga converter CO em acetato nas duas condições testadas (presença/ausência de sulfato), a utilização de CO foi mais rápida na presença de sulfato. A identificação dos microrganismos predominantes revelou a presença de organismos filogeneticamente próximos do género *Desulfotomaculum*.

A partir da cultura enriquecida em gás de síntese foi isolada uma nova bactéria: *Thermoanaerobacter carboxyditolerans*. Esta bactéria está filogeneticamente relacionada com *Thermoanaerobacter thermohydrosulfuricus* (97% similaridade, com base na sequência do gene 16S). *T. carboxyditolerans* não utiliza CO, mas tem a capacidade de crescer na presença de altas concentrações de CO (pCO = 1.7 bar). Outras espécies do género *Thermoanaerobacter* (*T. thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* e *T. wiegelii*) apresentaram também tolerância ao CO. O crescimento destas bactérias não foi significativamente afetado pela presença de CO em concentrações que variavam entre 25% a 100% de CO (pCO entre 0.425 e 1.7 bar). No entanto, verificou-se a diminuição da produção de hidrogénio, com o aumento da pressão parcial de CO no meio.

Uma nova bactéria termofílica, hidrogenogénica, carboxidotrófica, *Moorella stamsii*, foi isolada a partir da cultura enriquecida em CO. *M. stamsii* consegue converter o CO em hidrogénio. É filogenticamente próxima de *Moorella glycerini* (97% similaridade, com base na sequência do gene 16S).

Com os trabalhos de investigação decorrentes deste doutoramento, aumentamos os nossos conhecimentos relativamente à ecologia, fisiologia e microbiologia da conversão anaeróbia do gás de síntese e do monóxido de carbono, em condições termofílicas. Os resultados obtidos neste trabalho foram importantes, numa perspetiva futura de aplicar processos biotecnológicos para a produção de compostos com interesse, como o hidrogénio ou o acetato.

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Symbols and abbreviations

1,3 PDO	1,3 propanediol
ACS/CODH	carbon monoxide dehydrogenase acetyl co-A synthase complex
AQDS	anthraquinone-2,6-disulfonate
ARDRA	amplified ribosomal DNA-restriction analyse
ATP	adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
Bp	base pair
CAP	Contig Assembly Program
CFeSP	corrinoid iron-sulfur protein
CGMCC	China General Microbiological Culture Collection
CH_3COOH	acetate
CH_4	methane
CO	carbon monoxide
CO_2	carbon dioxide
CODH	carbon monoxide dehydrogenase
CR	carbon recovery
DDBJ	DNA DataBank of Japan
DGGE	denaturing gradient gel electrophoresis
DNA	desoxyribonucleic acid
DSMZ	German Collection of Microorganisms and Cell Cultures
EDTA	Ethylene-diamine-tetraacetic acid
EMBL	European Molecular Biology Laboratory
ENA	European Nucleotide Archive
GC	gas chromatography
H_2	hydrogen
H_2O	water
H_2S	hydrogen sulfide
HCN	hydrogen cyanide
HPLC	high-performance liquid chromatography
N_2	nitrogen
NCBI	National Center for Biotechnology Information

NH_3	ammonia
NOx	nitrogen oxides
PCR	polymerase chain reaction
RDP	ribossomal database project
rpm	revolutions per minute
rRNA	ribossomal ribonucleic acid
SO_2	sulfur dioxide
SO_4^{2-}	sulfate
SRB	sulfate reducing bacteria
SRP	sulfate reducing prokaryotes
Syn	syngas
VFA	volatile fatty acids
Vol, v	volume
Wt	weigth
$\Delta G0'$	standard free energy change

Chapter 1

Context, aim and thesis outline

In this chapter, anaerobic syngas fermentation is introduced as the subject of study. A brief overview of the main topic, focusing on the importance of syngas as a renewable source of energy and on the microbiology of the process, is given to contextualize the research. Finally, the research aim and scope of this thesis are presented.

1.1 Perspective and motivation

1.1.1 Syngas as a renewable source of energy

The replacement of fossil fuels by renewable energy sources is, nowadays, a worldwide priority, due to the price fluctuations and limited availability of petroleum. A variety of processes, both chemical and biological, for the production of biofuels has been the focus of scientific research and intensively studied since the last few years. Gasification processes and further bioconversion of synthesis gas (syngas) appears to be a promising alternative compared to the existing chemical techniques, since this process convert renewable sources into valuable chemical compounds and biofuels (Figure 1.1) (Hu et al., 2011; Jang et al., 2012).

Syngas is a gas mixture, mainly composed of CO, H_2 and CO_2, and is commonly produced from fossil fuels, but it can also be produced from a vast array of organic feedstocks such as lignocellulosic biomass and all kind of carbon-based waste (as coal, tar sands, etc), that cannot be directly converted to biofuels by microorganisms (Sipma et al., 2006).

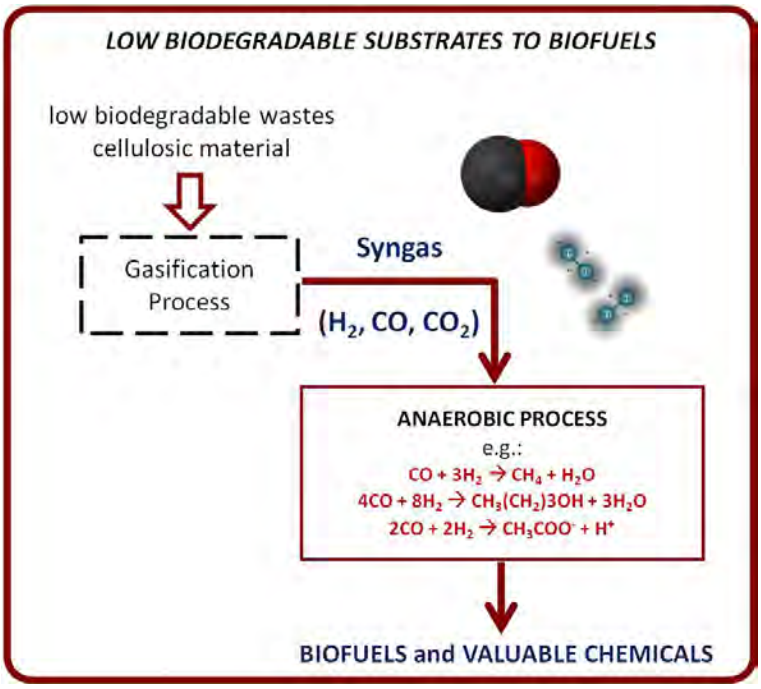


Figure 1.1: Schematic representation of the gasification process and further syngas bioconversion to biofuels and/or valuable chemicals.

The interest in the biological conversion of syngas relies on the knowledge that some anaerobic microorganisms can effectively use CO and H_2 to produce added-value carbon compounds, such as ethanol, butanol, methane, H_2, acetic acid or butyric acid (Bruant et al., 2010; Guiot et al., 2011; Henstra et al., 2007). The possibility of recovering the energy potential from biomass and wastes, is a strong motivation for studying processes with that purpose.

Ethanol is the biofuel mainly produced worldwide, and the majority of its production is from corn and sugar cane. On the other hand, butanol is nowadays considered a promising biofuel, but is also an important industrial chemical. Hydrogen is always considered as an ideal fuel for future transportation, since H_2 can be converted to energy with no CO_2 production. Since ethanol, butanol and hydrogen can be biologically produced from syngas and/or CO, the research on this field should be a priority.

Syngas fermentation to biofuels is a technology that is not yet commercially efficient, since it needs improvements, regarding to the bioreactor designing and/or the isolation of high yielding syngas/CO-rich waste gas degrading bacteria. Besides, there was one very good example of a company where this technology is already in operation: LanzaTech, in New Zealand. This company efficiently applies the process which converts carbon monoxide containing gases, as well as gases generated by gasification of forestry and agricultural residues, municipal waste, and coal, into valuable fuel and chemical products. The carbon monoxide containing gases are produced by different industries such as steel manufacturing, oil refining and chemical production.

1.1.2 Biological syngas fermentation

Since carbon monoxide is one of the main components of the syngas, carboxydotrophic microorganisms play an essential role on syngas and CO fermentation. These carboxydotrophic organisms are characterized by their ability to grow with CO as sole carbon and energy source. CO is metabolized by a wide variety of microorganisms, both mesophilic and thermophilic, from different physiological groups and isolated from a wide range of diverse habitats all around the world. The discovery of fast growing anaerobic bacteria capable of performing CO oxidation, presents an interesting alternative for the chemical catalytic process. The number of new syngas/CO-degrading bacteria that has been isolated, able to produce ethanol, butanol, hydrogen or methane, is increasing from the last few years (Henstra et al., 2007; Sipma et al., 2006; Sokolova et al., 2009). Currently, more attention has been done to the hydrogenogenic microorganisms, due to their ability to use CO and produce hydrogen. Most of them are thermophilic and efforts have been done to continue the research on this area, due to the importance of the thermophilic microorganisms on the overall process of syngas fermentation. In Table 1.1 there were presented some examples of well studied carboxydotrophic microorganisms, both mesophilic and thermophilic organisms.

1.2 Research Aim

The aim of the research presented in this thesis was to study in detail syngas fermentation by anaerobic mixed cultures under thermophilic conditions. For this propose, syngas and carbon monoxide were used as carbon and energy source during all the experiments. This thesis also outlines new insights on the microbiology of syngas and CO metabolism. New thermophilic

Table 1.1: Examples of microorganisms that can utilize syngas or CO as sole energy and carbon source.

Microorganism	T opt. (°C)	Products	References
Acetobacterium woodii	30	acetate	(Genthner and Bryant, 1987)
Butyribacterium methylotrophicum	37	etanol, butanol	(Lynd et al., 1982)
Clostridium autoethanogenum	37	ethanol, acetate	(Abrini et al., 1994)
Clostridium carboxidivorans	38	ethanol, butanol	(Liou et al., 2005)
Clostridium ljungdahlii	37	ethanol, acetate	(Tanner et al., 1993)
Carboxydothermus hydrogenoformans	70-72	hydrogen	(Svetlichny et al., 1991)
Desulfotomaculum carboxydivorans	55	hydrogen	(Parshina et al., 2005)
Moorella thermoacetica	58	acetate	(Collins et al., 1994; Daniel et al., 1990)
Moorella thermoautorophica	55	acetate	(Collins et al., 1994; Daniel et al., 1990)
Thermincola carboxydiphila	55	hydrogen	(Sokolova et al., 2005)
Methanosarcina barkeri	37	methane	(O'Brien et al., 1984)
Archaeoglobus fulgidus	83	acetate	(Henstra et al., 2007)
Methanothermobacter thermoautotrophicus	65	methane	(Daniels et al., 1977) (Wasserfallen et al., 2000)

microorganisms able to utilize or tolerate CO were also investigated.

1.3 Thesis outline

The focus of this thesis and its relevance in the framework of syngas fermentation was introduced in the present chapter.

A general overview on the current knowledge about biological conversion of syngas and CO is further present in **Chapter 2**. Emphasis is given to the microbiological aspects of syngas and CO fermentation.

In **Chapter 3**, different inocula were submitted to different temperature and substrate conditions, in order to obtain stable syngas- and CO-degrading cultures. The obtained cultures were further characterized by using molecular techniques.

A new *Thermoanaerobacter* species, *Thermoanaerobacter carboxyditolerans*, isolated from a syngas-degrading culture is presented in **Chapter 6**. Additionally, CO tolerance of different *Thermoanaerobacter* species was studied and is also presented in this chapter.

In **Chapter 5**, a new hydrogenogenic carboxydotrophic *Moorella* species, *Moorella stamsii*, is presented. This new strain was isolated from a CO-degrading culture and converts CO into H_2 and CO_2.

As an exploratory study, the syngas enriched culture obtained in Chapter 3 was used to evaluate the effect of sulfate on CO conversion by this culture. The preliminary results obtained in this work are described in **Chapter 4**.

Finally, **Chapter 7** summarizes the main conclusions. Perspectives for further research in this topic are also proposed.

Chapter 2

Fundamentals of biological syngas fermentation

Research on bioconversion of synthesis gas (or syngas) has demonstrated the potential to generate, biologically, fuels and chemicals from syngas. The interest of this conversion relies on the fact that gasification processes can be applied to a diverse range of feedstocks, including lignocellulosic and other recalcitrant materials. This chapter reviews current knowledge on biological syngas fermentation. First, a general overview about the gasification process, syngas production and syngas chemical composition is presented. Further on, a comprehensive review on currently isolated carboxydotrophic microorganisms is done and there potential involved in syngas and CO anaerobic degradation extensively discussed. A focus is given to the main microbial metabolic pathways involved in CO metabolism. Biotechnological applications and future perspectives and directions on syngas research regarding the technology and microbiology are also presented.

2.1 Introduction

2.1.1 Global energy crisis and biofuels production

Global energy crisis and limited supply of fossil fuels have renewed the worldwide focus on the development of sustainable processes and technologies for alternative fuel production. Energy production from renewable sources is a global priority. The European Union (EU) directive 2009/28/EC of the European Parliament and of the Council states that, for all state members, until 2020, 14% of fossil fuels used in transportation should be replaced by renewable energy sources (directive 2009/28/EC of 23 April 2009). Because biofuels are sustainable, renewable and environmentally friendly they have been considered an outstanding alternative to the use of fossil resources (Daniell et al., 2012; Jang et al., 2012; Tirado-Acevedo et al., 2010). The increasing demand for renewable feedstock-based biofuels is being the driving force for the rapid development of processes to produce fuels and chemicals. In this context, gasification is a good example of a process that can be used to convert, not only lignocellulosic feedstocks (agricultural and municipal wastes, wood, straw, grasses, crop residues, etc.), but also other recalcitrant and raw materials such as plastics, shale oil, coal, into synthesis gas (syngas), a gas mixture composed mainly by H_2, CO and CO_2 (Abubackar et al., 2011; Griffin and Schultz, 2012; Hu et al., 2011) (Figure 2.1). Gasification simplifies the substrate and syngas can be further utilized to generate fuels and chemicals (Figure 2.1). In this way, gasification process is considered an alternative to conventional fermentative pathways, constituting a more direct pathway towards the production of acetyl-CoA. Gasification process presents several advantages, namely feedstock and product flexibility, high efficiency and high energy conversion.

Syngas conversion can be chemical or biological, but the biological process offers several advantages over catalytic conversion that will be explained later in this chapter. The possibility of valorization and recovering the energy potential of biomass and recalcitrant wastes is a strong motivation for developing biological processes converting syngas.

Carbon monoxide is one of the main components of syngas. Its concentration in syngas can fluctuate from 5 to 60%, making it more difficult and restrictive for a direct industrial application of syngas. Although there are several biological reductive processes sensitive to the presence of high carbon monoxide concentrations, CO is considered a very important and potent electron donor in various redox reactions ($\Delta G^{0'} = -520\,mV$ for CO/CO_2 couple), providing energy for microbial metabolism (Sokolova et al., 2009). As also stated by Techtmann et al. (2009), CO is considered crucial within key metabolic functions and signal transduction in organisms from all three domains of life. Because CO is a major constituent of syngas, but also because CO occurs in a variety of environments due to anthropogenic and natural activity, it is of utmost importance to study the microorganisms and the processes in which these organisms convert CO into valuable chemicals and biofuels.

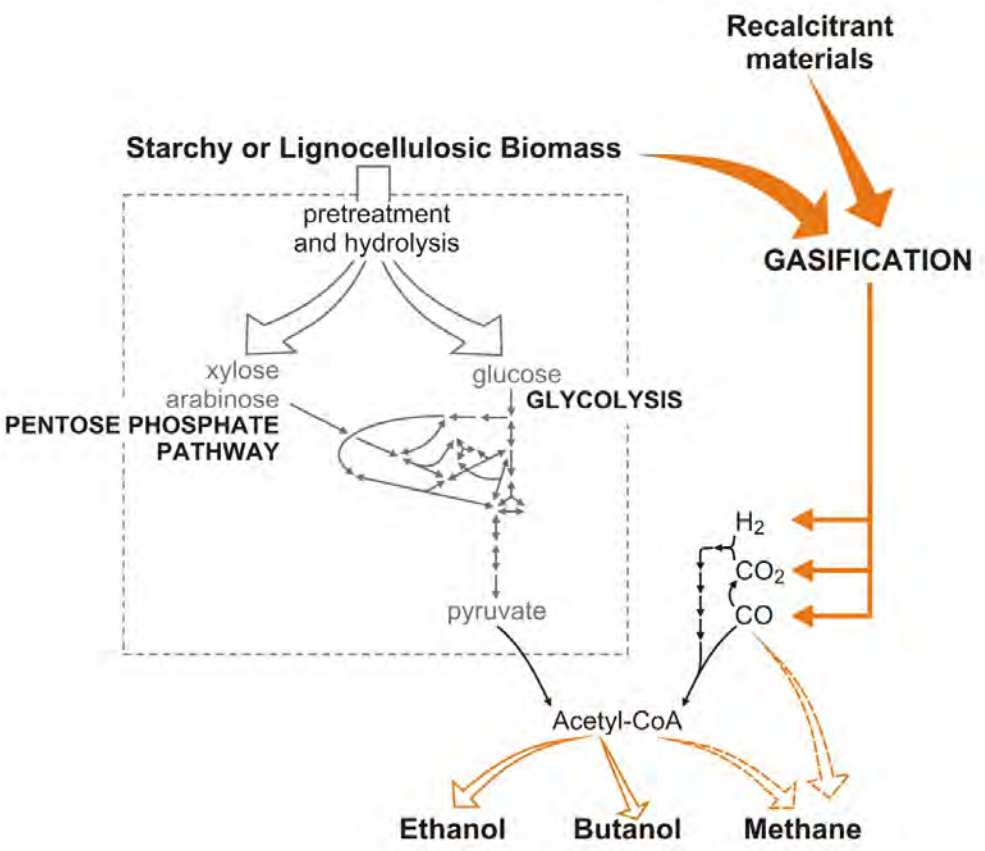


Figure 2.1: Schematic representation of the conversion of biomass and/or recalcitrant materials into biofuels.

2.1.2 Gasification, syngas production and syngas composition

Gasification is a thermochemical process of converting carbonaceous materials. It is performed at elevated temperatures (higher than 700 °C), with a controlled supply of oxygen, steam, air or supercritical water. Under these conditions, carbonaceous materials are converted into a gas mixture - syngas -, consisting mainly of CO, H_2, CO_2 and also CH_4 and N_2 (Abubackar et al., 2011; Daniell et al., 2012). It is commonly called direct gasification when air or oxygen is used as oxidant, originating exothermic reactions. Indirect gasification is the process where steam is used as oxidant, being characterized as an endothermic process, but thermodynamically more efficient (Sipma et al., 2006). Gasification occurs trough several successive reactions, namely drying, pyrolysis, combustion and reduction (Tirado-Acevedo et al., 2010). There are many different types of gasifiers classified according to the way air or oxygen is introduced in the system. The updraft (Figure 2.2-a)) and the downdraft (Figure 2.2-b)) types are the more common and simplest gasification reactors. The four process zones of gasification, and reactions occuring in the process are shown in figure 2.2, indicated by the numbers from 1 to 4.

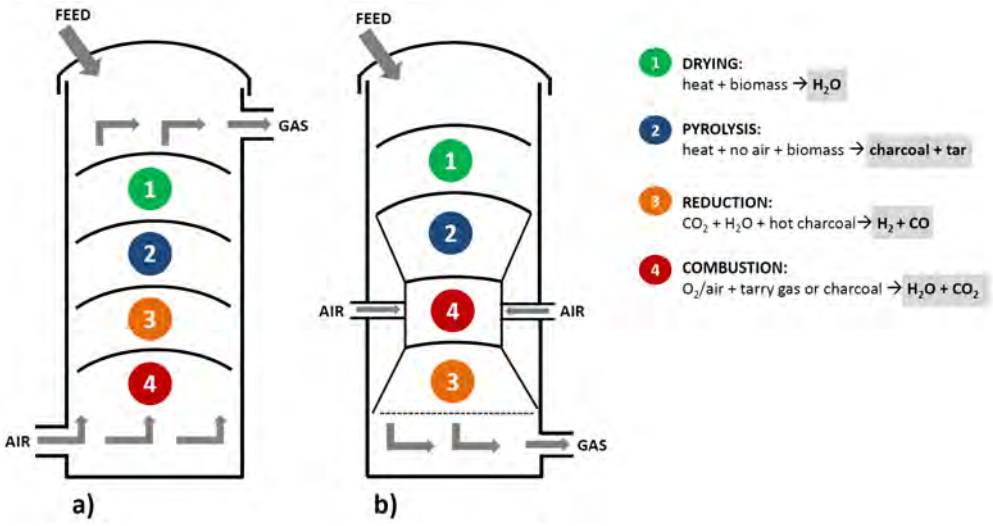


Figure 2.2: Diagrams of gasification reactors: a) updraft gasifier and b) downdraft gasifier.

Another type of gasifier that is a more recent configuration and does not have differential reaction zones is the fluidized-bed gasifier. This type of gasifier presents some advantageous over the updraft and downdraft gasifiers: uniformous temperature distribution in the fluid bed, carbon conversion up to 100% and is suitable for large-scale applications (Tirado-Acevedo et al., 2010).

Gasification is a flexible process in what concerns to feedstock materials. Different raw materials can be gasified, including coal, shale oil, tar sands, heavy residue and poorly degradable biomass sources (like straw and wood). The composition of syngas depends on several parameters such as feedstock properties (ash, moisture, particle size), gasifying agent (air, steam, pure oxygen or a combination of them), type of gasifier (fixed bed updraft or downdraft, fluidized bed, etc.) and operational conditions (temperature, gasifying agent to fuel ratio, pressure, etc.) (Abubackar et al., 2011; Mohammadi et al., 2011). For instance, coal-derived gas (most common substrate used for syngas production) is rich in CO and H_2, with lower concentrations of CO_2 and CH_4 and traces of H_2S and NH_3. In addition, if pure oxygen is used as oxidant in the gasifier, the resulting syngas is rich in CO and H_2; but, if air is used, syngas is a mixture of CO, CO_2, H_2, CH_4, N_2 and some light hydrocarbons, such as acetylene and ethylene (Sipma et al., 2006; Tirado-Acevedo et al., 2010). Concentration of CO in syngas increases with increased C to H ratio in the feedstock. The gasification of coal is usually simpler and less reactive than the gasification of solid waste and biomass, because of the diversity of the carbon-based materials present in that type of feedstock. Syngas produced from biomass gasification often contains impurities, namely acetylene, ethylene, ethane, benzene, hydrogen sulfide, ammonia, carbonyl sulfide, oxygen, mono-nitrogen oxides and also tars and ashes (Xu and Lewis, 2012). Although there are few studies on the potential inhibition of syngas fermentation by syngas impurities,

Table 2.1: Some examples of typical synthesis gas composition derived from different raw materials (adapted from Sipma et al. (2006) and Tirado-Acevedo et al. (2010)). Data are presented in descending order of CO percentage in the produced gas mixture.

	Composition (vol%)					
Source	**CO**	**CO_2**	**H_2**	**N_2**	**CH_4**	**Other**
Coal gasification	59.4	10.0	29.4	0.6	0.0	0.6
Partial oxidation of heavy fuel oil	47.0	4.3	46.0	1.4	0.3	1.0
Water gas	30.0	3.4	31.7	13.1	12.2	9.6
Pine wood chips	16.1	13.6	16.6	37.6	2.7	13.4
Natural gas, steam reforming	15.5	8.1	75.7	0.2	0.5	0.0
Switch grass	14.7	16.5	4.4	56.8	4.2	3.4
Demolition wood/paper residue	9.2	16.1	6.1	63.2	2.8	2.6
Dairy biomass	8.7	15.7	18.6	56.0	0.6	0.4
Cacao shells	8.0	16.0	9.0	61.5	2.3	3.2
Coke oven gas	5.6	1.4	55.4	4.3	28.4	4.9

it is known that microbial catalysts can manage better with syngas contaminants than some chemical catalysts (Xu et al., 2011). Table 2.1 represents typical composition of syngas produced from different sources.

2.1.3 Syngas: advantages of biological conversion

The production of fuels and chemicals, such as ethanol, butanol, acetate, butyrate, H_2, or CH_4, through syngas fermentation is considered to be more attractive and offers several advantages over metal catalytic conversion (Henstra et al., 2007; Munasinghe and Khanal, 2010; Worden et al., 1997). Although the biological processes tend to be slower than chemical reactions, there are some main advantages to syngas fermentation: higher specificity of the biocatalyst, lower energy costs, lower purification costs, elimination of complex pretreatment steps and costly enzymes, greater resistance to catalyst poisoning, independence of a fixed H_2:CO ratio for bioconversion, and aseptic operation of syngas fermentation due to generation of syngas at high temperatures (Heiskanen et al., 2007; Henstra et al., 2007; Munasinghe and Khanal, 2010; Tirado-Acevedo et al., 2010). Regarding catalyst poisoning, a good example to emphasize is the hydrogen sulfide-gas. In a biological process, it would not be required to remove the hydrogen sulfide, since the microorganisms involved are fairly tolerant towards sulfide, being able to grow in the presence of up to 2% of hydrogen sulfide (Henstra et al., 2007; Tirado-Acevedo et al., 2010). In addition, gas fermentation has great feedstock flexibility and high rates of energy and carbon capture. The irreversible character of biological reactions also allows complete conversion and avoids thermodynamic equilibrium relations (Daniell et al., 2012; Klasson et al., 1992). Table 2.2 summarizes the main reactions with syngas components during anaerobic syngas fermentation.

Syngas fermentation is possible due to the existence of many microorganisms available that

Table 2.2: Microbial reactions and energetics during CO/syngas anaerobic conversion (adapted from Sipma et al. (2006)). $\Delta G^{0'}(55°C)$ was calculated using thermodynamic data according to Hanselmann (1991).

Products		$\Delta G^{0'}(25°C)$ kJ reaction^{-1} **(pH 7.00)**	$\Delta G^{0'}(55°C)$ kJ reaction^{-1} **(pH 6.58)**
From CO			
formate	$CO + H_2O \longrightarrow HCOO^- + H^+$	-16	-17
acetate	$4\,CO + 4\,H_2O \longrightarrow CH_3COO^- + 2\,HCO_3^- + 3\,H^+$	-166	-177
butyrate	$10\,CO + 4\,H_2O \longrightarrow CH_3(CH_2)_2COO^- + H^+ + 6\,CO_2$	-440	-454
ethanol	$6\,CO + 3\,H_2O \longrightarrow CH_3CH_2OH + 4\,CO_2$	-222	-232
n-butanol	$12\,CO + 5\,H_2O \longrightarrow CH_3(CH_2)_3OH + 8\,CO_2$	-480	-497
hydrogen	$CO + H_2O \longrightarrow CO_2 + H_2$	-20	-18
methane	$4\,CO + 2\,H_2O \longrightarrow 3\,CO_2 + CH_4$	-211	-214
From H_2/CO			
acetate	$2\,CO + 2\,H_2 \longrightarrow CH_3COO^- + H^+$	-134	-145
butyrate	$4\,CO + 6\,H_2 \longrightarrow CH_3(CH_2)_2COO^- + H^+ + 2\,H_2O$	-240	-271
methanol	$CO + 2\,H_2 \longrightarrow CH_3OH$	-39	-46
ethanol	$2\,CO + 4\,H_2 \longrightarrow CH_3CH_2OH + H_2O$	-288	-292
n-butanol	$4\,CO + 8\,H_2 \longrightarrow CH_3(CH_2)_3OH + 3\,H_2O$	-324	-354
methane	$CO + 3\,H_2 \longrightarrow CH_4 + H_2O$	-151	-158
From H_2/CO_2			
acetate	$2\,HCO_3^- + 4\,H_2 + H^+ \longrightarrow CH_3COO^- + 4\,H_2O$	-104	-115
methane	$HCO_3^- + 4\,H_2 + H^+ \longrightarrow CH_4 + 3\,H_2O$	-135	-143
From acetate			
methane	$CH_3COO^- + H_2O \longrightarrow CH_4 + HCO_3^-$	-31	-28

convert CO and H_2 (the main components of syngas, table 2.1) into multicarbon compounds. Although most organisms that have been shown to form organic compounds from syngas are mesophilic, growth at high temperatures is advantageous, as less cooling of the syngas is required before it is introduced into the bioreactor (Henstra et al., 2007). Additionally, higher temperatures can lead to higher conversion rates and benefit separation of the product. Besides the main disadvantage of a thermophilic process, i.e., the lower solubility of CO and H_2 at higher temperatures that can impact negatively the process efficiency, the benefit in product recovery improves the overall cost effectiveness of the process (Henstra et al., 2007). Furthermore, there were several attempts to utilize also extreme thermophiles (optimum growth temperature > 70 °C) to produce organic solvents and biofuels (Henstra and Stams, 2011).

2.2 Biological syngas conversion

2.2.1 Biochemistry of gas fermentation: the Wood-Ljungdahl pathway

Anaerobic microorganisms can be used as biocatalysts to produce a variety of valuable metabolites from syngas. These products include acetic, propionic and butyric acid, as well as methanol, ethanol and butanol, and also methane and hydrogen (Abubackar et al., 2011). For the production of those compounds from syngas, syngas- and CO-fermenting microorganisms use the Wood-Ljungdahl pathway, also known as reductive acetyl-CoA pathway. The reductive acetyl-CoA pathway is made up of two branches: the methyl branch, sometimes referred to as "eastern" and the carbonyl branch, also known as "western" branch (Ragsdale, 1997). CO and/or CO_2 feeds the methyl and carbonyl branches of the pathway, with the formation of acetyl coenzyme A (acetyl-CoA) (Figure 2.3). The reductive acetyl-CoA pathway is an irreversible and non-cyclic pathway that evolved in strict anaerobic conditions (Munasinghe and Khanal, 2010).

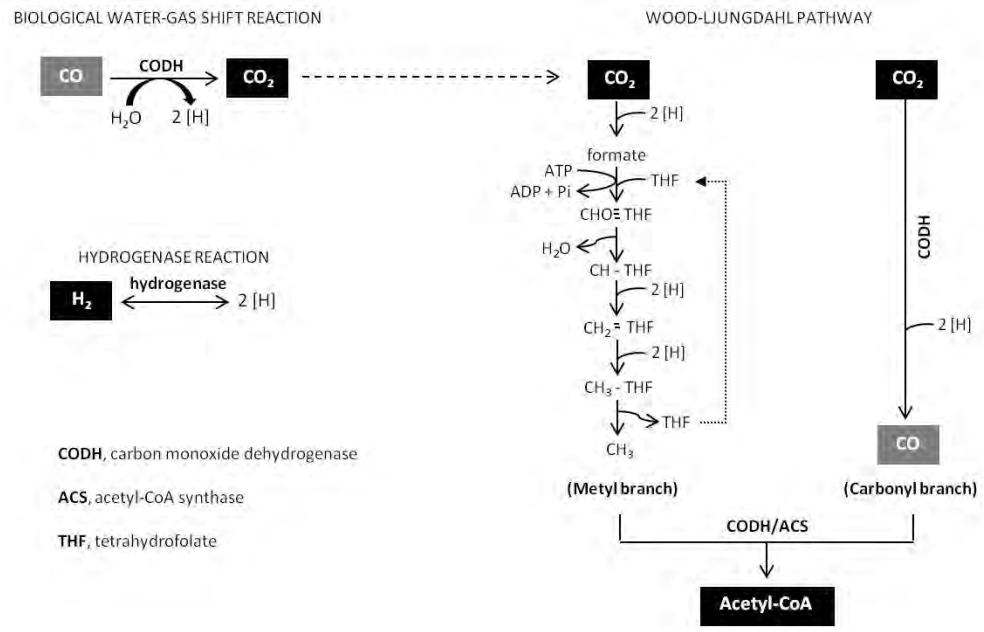


Figure 2.3: Simplified scheme of Wood-Ljungdahl pathway (adapted from Köpke et al. (2011)).

This pathway was discovered and unraveled by Wood and Ljungdahl (Ljungdahl and Wood, 1969). At that time, these two authors proposed a scheme for the synthesis of acetate from CO_2 by the acetogenic bacterium *Moorella thermoacetica* (f. *Clostridium thermoaceticum*) (Collins et al., 1994; Daniell et al., 2012; Fontaine et al., 1942). The pathway is present in bacteria as well as in archaea, with minor differences. The biochemistry of this pathway has been comprehensively described in many reviews (Ljungdhal, 1986; Ragsdale, 2004; Ragsdale and Pierce, 2008; Wood, 1991). The first step of syngas conversion involves the conversion of CO and/or CO_2 to two-carbon acetyl-CoA intermediates trough two different branches. In the methyl branch (or

eastern), one CO_2 molecule is converted to a methyl moiety. In this branch, the enzyme formate dehydrogenase (FDH) starts the reactions by reducing CO_2 to formate, which is then attached to tetrahydrofolate (THF) by 10-formyl-THF synthetase. The successive several reductive steps are catalyzed by enzymes including: methylene-THF cyclohydrolase (MTC), methylene-THF dehydrogenase (MTD) and methylene-THF reductase (MTRS) (Daniell et al., 2012; Henstra et al., 2007; Hu et al., 2011; Munasinghe and Khanal, 2010). Methyltransferase (MTR) is responsible for the last step, transferring the methyl group from methyl-THF to a corrinoid-FeS protein (CFeSP), and then this methyl group is provided as the methyl group of acetyl-CoA. In the carbonyl branch (or western), two protons (or reducing equivalents) are utilized to reduce one CO_2 molecule to CO by a so called monofunctional carbon monoxide dehydrogenase (CODH) enzyme (Daniell et al., 2012; Henstra et al., 2007; Hu et al., 2011; Munasinghe and Khanal, 2010). CO is then incorporated in the methyl moiety to form acetyl-CoA via the acetyl-CoA synthase enzyme (ACS). CO can also directly enter the carbonyl branch to form acetyl-CoA directly via the CODH/ACS enzyme complex. Special attention is given to the carbonyl branch because this branch is unique for anaerobic microorganisms (Ragsdale, 1997). Furthermore, reducing equivalents required for metabolic processes are generated from either CO, via the CODH enzyme, or H_2, via hydrogenase enzyme (Köpke et al., 2011). In the absence of H_2 in the input gas, the reducing equivalents are provided exclusively from CO by the biological water-gas shift reaction. If H_2 is present in the gas, additional reducing equivalents are available, enabling a proportional increase in carbon assimilation. Acetyl-CoA formed is further reduced to acetate, ethanol or other byproducts.

2.2.1.1 Carbon monoxide dehydrogenase (CODH) and Acetyl-CoA synthase (ACS)

CODH and ACS enzymes are linked to the Wood-Ljungdahl pathway which plays a central role in CO metabolism. Utilization of CO by anaerobes is catalyzed by CODH and ACS. CODH and CODH/ACS complexes are widespread among anaerobes, both bacteria and archaea, and are found in methanogens, acetogens and sulfate reducers (Fischer et al., 2008; Sokolova et al., 2009). CODHs in anaerobic microorganisms are nickel containing, O_2 sensitive enzymes and can be monofunctional or bifunctional.

The monofunctional CO dehydrogenase (CODH) is responsible to oxidize CO to CO_2 and H_2 through a water-gas shift reaction: $CO_2 + 2\,H^+ + 2\,e^- \longleftrightarrow CO + H_2O$. The monofunctional CODH functions in the direction of CO oxidation, allowing microbes to take up and oxidize CO even at low levels.

The multi-subunit bifunctional metaloenzyme CO dehydrogenase (CODH)/Acetyl-CoA synthase (ACS), usually called CODH/ACS complex, is capable of both reducing CO_2 to CO, and accepting the methyl group of the eastern branch and condensing both the methyl and the carbonyl moiety with a CoA group to produce a molecule of coenzyme-A. CODH in the bifunctional protein converts CO_2 into acetyl-CoA, first with the formation of CO and secondly synthesizing

acetyl-CoA:

$CO_2 + 2\,H^+ + 2\,e^- \longleftrightarrow CO + H_2O$

and

$CO + CH_3{-}CFeSP + CoA \longrightarrow acetyl{-}CoA + CFeSP.$

2.2.2 Microbiology of gas fermentation

Microorganisms can be categorized into distinct groups depending on their optimum growth temperature, namely, psychrophiles, mesophiles, thermophiles and hyperthermophiles. Currently known microorganisms capable of fermenting syngas/CO-rich gas are mesophilic and thermophilic. In general, optimum growth temperature for mesophilic microorganisms varies from 37 to 40 °C, for thermophiles the temperature range varies from 55 to 80 °C. Until recently, only mesophilic microorganisms were known, but the number of new thermophiles discovered that have this ability has increased. Additionally, the recently isolated carboxydotrophic thermophiles are predominantly hydrogenogens, characterized by growing chemolithoautotrophically through conversion of CO and H_2O to H_2 and CO_2 (Henstra et al., 2007; Svetlitchnyi et al., 2001).

Thus far, biological syngas and CO conversion has been mainly studied using pure cultures or defined co-cultures (Bruant et al., 2010; Heiskanen et al., 2007; Köpke et al., 2010; Kundiyana et al., 2011; Parshina et al., 2005; Shen et al., 1999) and strongly directed to ethanol production (Abrini et al., 1994; Datar et al., 2004; Maddipati et al., 2011; Najafpour and Younesi, 2006; Wilkins and Atiyeh, 2011). However, other products such as butanol, acetic acid, butyric acid, hydrogen and methane can be obtained from syngas, as already referred (Bruant et al., 2010; Guiot et al., 2011; Henstra et al., 2007). Several mesophilic anaerobic microorganisms, e.g. *Clostridium carboxidivorans* and *Butyribacterium methylotrophicum*, were shown to produce short-chain fatty-acids and alcohols from CO and H_2. Thermophilic carboxydotrophic hydrogenogenic bacteria, e.g. *Carboxydothermus hydrogenoformans*, *Carboxydocella thermoautotrophica* and *Desulfotomaculum carboxydivorans*, can convert CO and H_2O to H_2 and CO_2. Direct conversion of CO to CH_4 can be achieved by a few methanogenic archaea, such as *Methanosarcina barkeri*, *Ms acetivorans* and *Methanothermobacter thermoautotrophicus*.

Mixed culture approaches for the conversion of these substrates have received little attention. Sipma and coworkers have studied batch CO conversion by anaerobic mesophilic and thermophilic sludges to acetate and/or hydrogen (Sipma et al., 2003, 2004). Other research groups observed hydrogenotrophic-linked methane production during continuous CO conversion by mixed communities in mesophilic and thermophilic bioreactors (Guiot et al., 2011).

Tables 2.3, 2.4 and 2.5 give an overview of the strictly anaerobic microorganisms from mesophilic and thermophilic groups, known to utilize syngas/CO as carbon and energy source. Characteristics of these microorganisms such as, optimal temperature and pH for growth, origin of isolates and main products formed from syngas/CO fermentation are also shown (ta-

bles 2.3, 2.4 and 2.5). Carboxydotrophic organisms are distributed in both bacterial and archaeal domains (Figures 2.4 and 2.5) and were isolated from a variety of different locations, meaning that anaerobic carboxydotrophs are widespread in nature and man-made ecosystems. The bacterial species belong mainly to Firmicutes phylum. There are also some microorganisms from Proteobacteria and Dictyoglomi phyla. The majority of the bacterial carboxydotrophic species belong to Clostridia class, although there are some representatives from other classes, namely Deltaproteobacteria, Dictyoglomia, Thermolithobacteria and Negativicutes. Regarding to the archaeal carboxydotrophic microorganisms, they are all from the same phylum, Euryarchaeota, with representatives from four different classes: Methanobacteria, Methanomicrobia, Archaeoglobi and Thermococci.

How widespread microorganisms are that are capable of using CO is still unclear. There are some facts suggesting that the number of carboxydotrophic organisms is largely underestimated because i) CO utilization is not commonly tested in growth studies; ii) CO may initially inhibit growth and adaptation to CO is needed, which only occurs after long term incubation or after multiple transfers with increasing CO levels; and iii) the presence of CO oxidizing microorganisms in habitats, such as anaerobic bioreactor sludge, where CO does not seem to be present, but may be intermediately formed, has not been explored (Sipma et al., 2006).

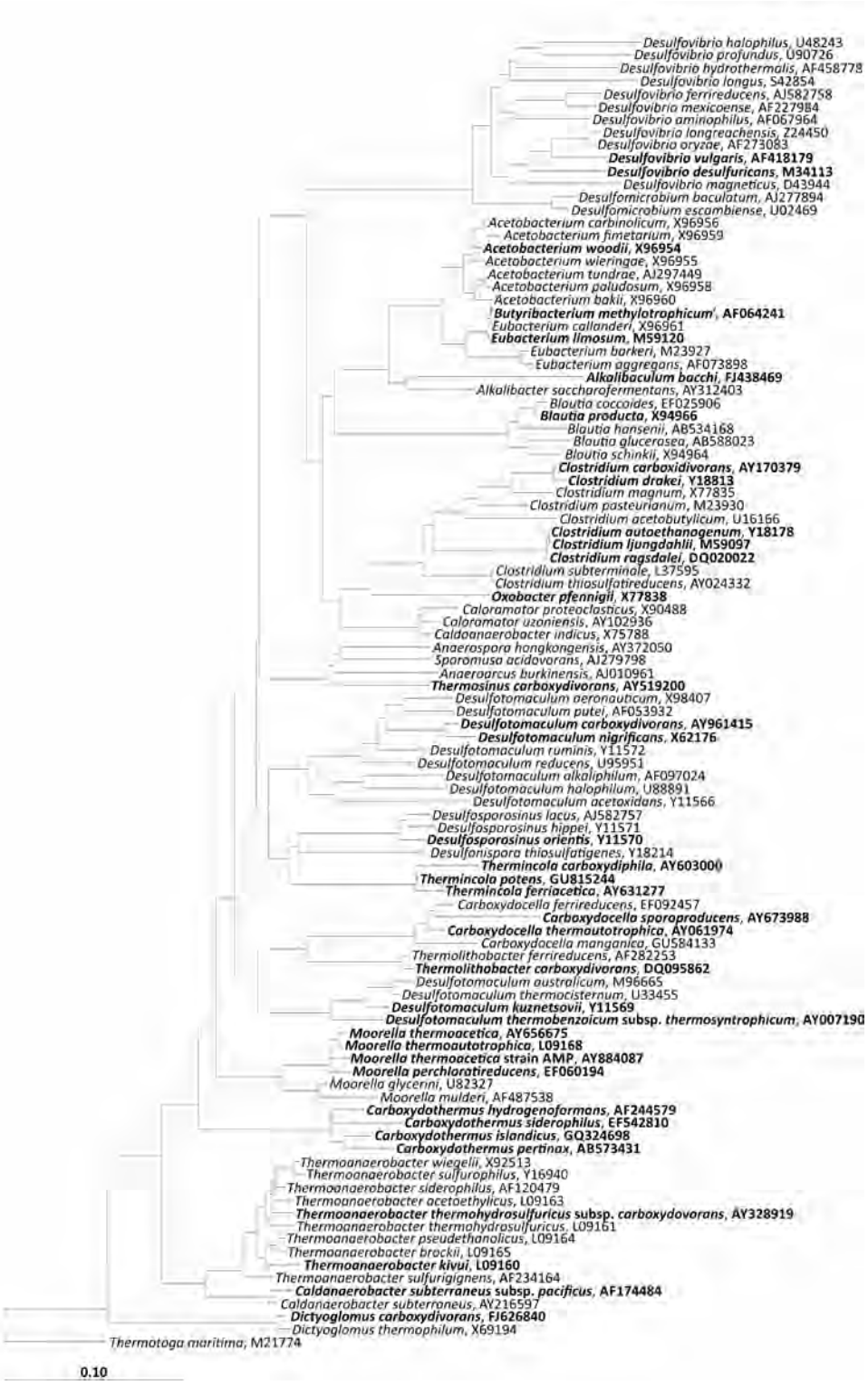


Figure 2.4: 16S rRNA gene sequenced-based phylogenetic tree of anaerobic carboxydotrophic **bacterial** species. The phylogenetic tree was calculated using the ARB software package (Saitou and Nei, 1987) and applying the neighbor-joining method. GenBank accession numbers of 16S rRNA gene sequences are indicated. The scale bar indicates the branch length that represents 10% sequence dissimilarity.

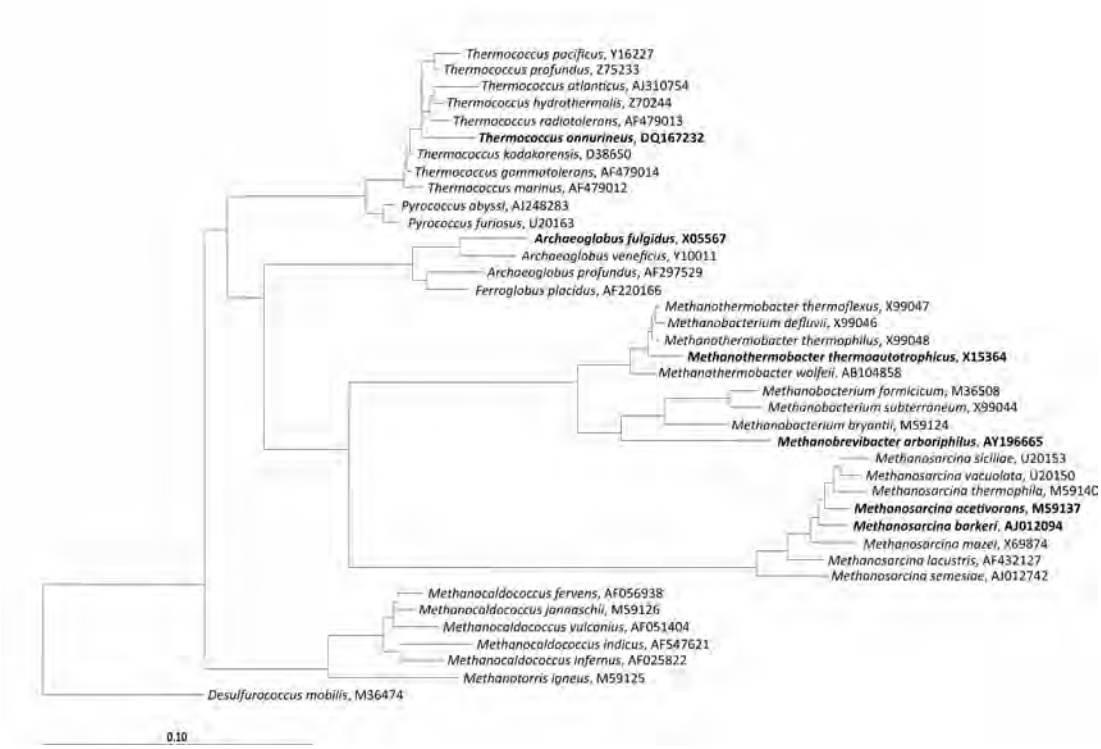


Figure 2.5: 16S rRNA gene sequenced-based phylogenetic tree of anaerobic carboxydotrophic **archaeal** species. The phylogenetic tree was calculated using the ARB software package (Saitou and Nei, 1987) and applying the neighbor-joining method. GenBank accession numbers of 16S rRNA gene sequences are indicated. The scale bar indicates the branch length that represents 10% sequence dissimilarity.

Table 2.3: Overview of **mesophilic** anaerobic carboxydotrophic microorganisms (n.r., not reported).

Microorganism	Domain/Phylum	T opt. (°C)	pH opt.	Products	Origin	References
Acetobacterium woodii	Bacteria/Firmicutes	30	6.8	acetate	marine sediments	(Genthner and Bryant, 1987)
Alkalibaculum bacchi	Bacteria/Firmicutes	37	8.0 – 8.5	acetate, ethanol	livestock-impacted soil	(Allen et al., 2010)
Blautia producta	Bacteria/Firmicutes	37	7.0	acetate	human septicemia	(Liu et al., 2008; Lorowitz and Bryant, 1984)
Butyribacterium methylotrophicum	Bacteria/Firmicutes	37	6.0	acetate, ethanol, butyrate, butanol	sewage sludge digestor	(Lynd et al., 1982)
Clostridium autoethanogenum	Bacteria/Firmicutes	37	5.8 – 6.0	ethanol, acetate	rabbit feces	(Abrini et al., 1994)
Clostridium carboxidivorans	Bacteria/Firmicutes	38	6.2	ethanol, acetate	agricultural settling lagoon	(Liou et al., 2005)
Clostridium drakei	Bacteria/Firmicutes	30 – 37	5.5 – 7.5	ethanol, acetate	acidic coal-mine pond	(Küsel et al., 2000; Liou et al., 2005)
Clostridium ljungdahlii	Bacteria/Firmicutes	37	6.0	ethanol, acetate	chicken yard waste	(Tanner et al., 1993)
Clostridium ragsdalei	Bacteria/Firmicutes	30 – 35	6.0	ethanol, acetate	duck pond sediment	(Kundiyana et al., 2011)
Desulfosporosinus orientis	Bacteria/Proteobacteria	30 – 37	7.0	H_2S, CO_2	soil	(Stackebrandt et al., 1997)
Desulfovibrio desulfuricans	Bacteria/Proteobacteria	37	n.r.	H_2, CO_2, H_2S	oil well corrosion site	(Davidova et al., 1994)
Desulfovibrio vulgaris	Bacteria/Proteobacteria	37	n.r.	H_2, CO_2, H_2S	soil	(Lupton et al., 1984)
Eubacterium limosum	Bacteria/Firmicutes	38 – 39	7.0 – 7.2	acetate	rumen fluid	(Genthner and Bryant, 1987)
Oxobacter pfennigii	Bacteria/Firmicutes	36 – 38	7.3	acetate, n-butyrate	rumen of cattle	(Collins et al., 1994; Krumholz and Bryant, 1985)
Methanobrevibacter arboriphilicus	Archaea/Euryarchaeota	35 – 40	6.0 – 7.5	methane	soil	(Hammel et al., 1984)
Methanosarcina acetivorans strain C2A	Archaea/Euryarchaeota	37	7.0	acetate, formate methan	marine mud	(Rother and Metcalf, 2004)
Methanosarcina barkeri	Archaea/Euryarchaeota	37	7.4	methane	anaerobic sewage digester	(O'Brien et al., 1984)

Table 2.4: Overview of **thermophilic** anaerobic carboxydotrophic microorganisms; (n.r., not reported).

Microorganism	**Domain/Phylum**	**T opt.** (°C)	**pH opt.**	**Products**	**Origin**	**References**
Caldanaerobacter subterraneus subsp. *pacificus*	Bacteria/Firmicutes	70	6.8 – 7.1	hydrogen	submarine hot vent	(Sokolova et al., 2001)
Carboxydocella sporoproducens	Bacteria/Firmicutes	60	6.8	hydrogen	Karymskoe Lake	(Slepova et al., 2006)
Carboxydocella thermoautotrophica	Bacteria/Firmicutes	58	7.0	hydrogen	Geyser Valley	(Sokolova et al., 2002)
Carboxydothermus hydrogenoformans	Bacteria/Firmicutes	70 – 72	6.8 – 7.0	hydrogen	Kunashir, Kuril Islands	(Svetlichny et al., 1991)
Carboxydothermus islandicus	Bacteria/Firmicutes	65	5.5 – 6.0	hydrogen	icelandic hot spring	(Novikov et al., 2011)
Carboxydothermus pertinax	Bacteria/Firmicutes	65	5.5 – 6.0	hydrogen	volcanic acidic hot spring	(Yoneda et al., 2012)
Carboxydothermus siderophilus	Bacteria/Firmicutes	65	6.5 – 7.2	hydrogen	Geyser Valley	(Slepova et al., 2009)
Desulfotomaculum carboxydivorans	Bacteria/Firmicutes	55	7.0	hydrogen, H_2S	anaerobic bioreactor	(Parshina et al., 2005)
Desulfotomaculum kuznetsovii	Bacteria/Firmicutes	60	7.0	acetate, H_2S	underground thermal mineral water	(Parshina et al., 2005)
Desulfotomaculum nigrificans	Bacteria/Firmicutes	55	7.0	hydrogen, H_2S	sewage mud, soil	(Parshina et al., 2010)
Desulfotomaculum thermobenzoicum ssp. *thermosyntrophicum*	Bacteria/Firmicutes	55	7.0	acetate, H_2S	anaerobic sludge	(Parshina et al., 2005; Plugge et al., 2002)
Dictyoglomus carboxydivorans	Bacteria/Dictyoglomi	80	6.5	hydrogen	Uzon Caldera	(Kochetkova et al., 2011)
Moorella perchloratireducens	Bacteria/Firmicutes	55 – 60	7.0	acetate	underground gas storage	(Balk et al., 2008)

Table 2.5: Overview of **thermophilic** anaerobic carboxydotrophic microorganisms (cont.); (n.r., not reported).

Microorganism	**Domain/Phylum**	**T opt.** (°C)	**pH opt.**	**Products**	**Origin**	**References**
Moorella strain AMP	Bacteria/Firmicutes	60 – 65	6.9	hydrogen	anaerobic bioreactor	(Jiang et al., 2009)
Moorella thermoacetica	Bacteria/Firmicutes	55	6.5 – 6.8	acetate	horse feces	(Collins et al., 1994; Daniel et al., 1990)
Moorella thermoautorophica	Bacteria/Firmicutes	58	6.1	acetate	mud and water from hot spring	(Collins et al., 1994; Savage et al., 1987)
Thermincola carboxydiphila	Bacteria/Firmicutes	55	8.0	hydrogen	Bolshaya river	(Sokolova et al., 2005)
Thermincola ferriacetica	Bacteria/Firmicutes	57 – 60	7.0 – 7.2	hydrogen	Kuril Islands	(Zavarzina et al., 2007)
Thermincola potens	Bacteria/Firmicutes	n.r.	n.r.	hydrogen	thermophilic microbial fuel cell	(Byrne-Bailey et al., 2010)
Thermoanaerobacter kivui	Bacteria/Firmicutes	60	n.r.	acetate	mud, lake Kivu	(Kevbrina et al., 1996)
Thermoanaerobacter thermohydrosulfuricus subsp. *carboxydovorans*	Bacteria/Firmicutes	70	6.3 – 6.8	hydrogen	geothermal spring	(Balk et al., 2009)
Thermolithobacter carboxydivorans	Bacteria/Firmicutes	70	7.0	hydrogen	Raoul Island	(Sokolova et al., 2007)
Thermosinus carboxydivorans	Bacteria/Firmicutes	60	6.8 – 7.0	hydrogen	Norris Basin	(Sokolova et al., 2004)
Archaeoglobus fulgidus	Archaea/Euryarchaeota	83	6.4	acetate, formate, H_2S	submarine hot spring	(Henstra et al., 2007; Stetter, 1988)
Methanothermobacter thermoautotrophicus	Archaea/Euryarchaeota	65	7.4	methane	sewage sludge	(Daniels et al., 1977; Wasserfallen et al., 2000)
Thermococcus strain AM4	Archaea/Euryarchaeota	82	6.8	hydrogen	deep-sea hydrothermal vent	(Sokolova et al., 2004)
Thermococcus onnurineus	Archaea/Euryarchaeota	80	8.5	hydrogen	deep-sea hydrothermal vent	(Bae et al., 2012)

2.2.2.1 Syngas/CO conversion by microbial anaerobic mixed cultures

The use of anaerobic mixed cultures in biological processes presents some advantages over the use of pure cultures. Despite the potential competition between the different groups of microorganisms, working with anaerobic mixed cultures gives an opportunity to study a more diverse and complex net of reactions or preferential pathways.

Most of the studies thus far have focused on the utilization of pure cultures or defined co-cultures to study syngas and CO conversion. Mixed culture approaches for the conversion of these substrates have received little attention and few reports are available on this subject.

Sipma and coworkers have contributed significantly to our understanding of syngas conversion by anaerobic mixed cultures, both at mesophilic and thermophilic conditions, and in batch tests or in continuous reactors (Sipma et al., 2003, 2006, 2004, 2007).

Sipma et al. tested six mesophilic anaerobic sludges from wastewater treatment reactors for their ability to convert CO at 30 and 55 °C. All the tested sludges could convert CO in the batch tests at 30 °C, with a CO depletion rate between 0.14-0.62 mmol CO day^{-1}. Conversion of CO at 55 °C was achieved by 5 sludges and CO depletion rates varied between 0.73-1.32 mmol CO day^{-1}. Sludges incubated at 30 °C produced methane and/or acetate, while incubation at 55 °C resulted in the formation of mainly methane and/or hydrogen. It was also observed that CO conversion at thermophilic conditions was significantly faster, although the tested sludges were not acclimated to high temperature.

Similar results were obtained by Guiot et al. (2011) during batch incubation of mesophilic anaerobic sludge: methane production in thermophilic (60 °C) assays was 5-fold higher than in the mesophilic (30 °C) incubations. Continuous CO conversion to methane has been also shown in a 30 L gas-lift reactor inoculated with anaerobic granular sludge from an industrial wastewater treatment plant. A maximum CO conversion of 75% was obtained for a CO partial pressure of 0.6 atm and a gas recirculation ratio of 1:20. Under these conditions, methane yield (CH_4/CO) was approximately 95% and other metabolites were formed only in trace amounts.

Kim and Chang (2009) and Hussain et al. (2011) have performed studies that coupled CO or syngas conversion with electricity production by using a microbial fuel cell (MFC). In the first study, by Kim & Chang, a fermenter was inoculated with anaerobic digester fluid from a municipal wastewater treatment plant and was fed with pure CO as the only substrate. The dominant microorganisms that were enriched in this mixed culture were identified and were related to the *Acetobacterium* genus. Under mesophilic conditions (30 °C) CO was converted into mainly acetate at a conversion rate of 1.26± 0.07 mmol h^{-1}. In this work a CO fermenter was enriched to produce volatile fatty acids, mainly acetate, and the products were fed to a MFC to generate electricity. The work from Hussain et al. (2011) describes the operation of an MFC, at 50 °C, with syngas as the sole electron donor. An anaerobic granular sludge from an agricultural waste degrading reactors was used as inoculum. Syngas conversion efficiency of 87 to 98% was obtained. In the first step syngas was converted to acetate that was further

consumed by the anodophilic bacteria to produce electricity.

Most of the anaerobic mixed cultures used for syngas or CO fermentation studies originate from wastewater treatment plants or full scale anaerobic reactors from industries. Biomass from these sources are excellent inocula, since it is cheap, easy to get and biologically diverse, containing microorganisms from both domains, Bacteria and Archaea. Another strategy was followed by Kochetkova et al. (2011). They studied CO conversion by microbial communities inhabiting three Kamchatka hot springs. CO is one of the common compounds found in natural thermal environments. Although the dissolved CO concentrations in the hot springs were low, this work confirmed the presence of CO-oxidizing prokaryotes and active CO conversion. The dominant process observed was hydrogenogenic CO conversion. Furthermore, from the samples of sediment and water collected in the hot springs, two strains of anaerobic thermophilic hydrogenogenic bacteria were isolated: *Carboxydocella* sp. 1503 and *Dictyoglomus* sp. 1512.

Working with mixed cultures opens new perspectives for the discovery of new carboxydotrophic organisms, contributing to the knowledge on the diversity of CO utilizing microorganisms.

2.2.2.2 Syngas/CO conversion by acetogenic bacteria

Acetogenic bacteria use the acetyl-CoA pathway as their major means of generating energy for growth (Ragsdale and Pierce, 2008). Acetate synthesis from CO in acetogenic bacteria is a result of the following reaction: $4\,CO + 2\,H_2O \longrightarrow CH_3COOH + 2\,CO_2$ (Drake and Daniel, 2004). Acetogenic bacteria can also use CO to produce ethanol, butanol and other products. When acetogens grow on H_2/CO_2 carbon enters the Wood-Ljungdahl pathway at the CO_2 reduction step (Figure 2.3), with H_2 serving as the electron donor. When growing on CO, CODH catalyzes formation of CO_2, which is used in the methyl branch of the pathway, and CO is directly incorporated as the carbonyl group of acetyl-CoA (Ragsdale and Pierce, 2008).

Acetogens have been isolated from a variety of habitats including soil, sediments, intestinal tracts of animals and humans, and are found worldwide. All the acetogens isolated to date produce acetate and the majority of the isolated microorganisms are homoacetogens. *Clostridium ljungdahlii*, *C. carboxydivorans*, *C. ragsdalei*, *Alkalibaculum bacchi*, *C. autoethanogenum*, *C. drakei* and *Butyribacterium methylotrophicum* are some examples of homoacetogens that are able to produce acetate, but also ethanol from carbon monoxide (Abubackar et al., 2011).

Butyribacterium methylotrophicum is an example of an anaerobe capable to use 1-carbon compounds such as CO_2, methanol and formate, but also has the ability to produce ethanol from syngas. This microorganism is one of the most versatile CO-utilizing acetogen (Zeikus et al., 1983).

Clostridium ljungdahlii was the first acetogenic microorganism shown with the ability of converting syngas to ethanol (Tirado-Acevedo et al., 2010). With an optimum temperature for growth between 37 and 40 °C, and a pH of $5.8-6.0$ (Tanner et al., 1993) (Figure 2.6-a). *Clostrid-*

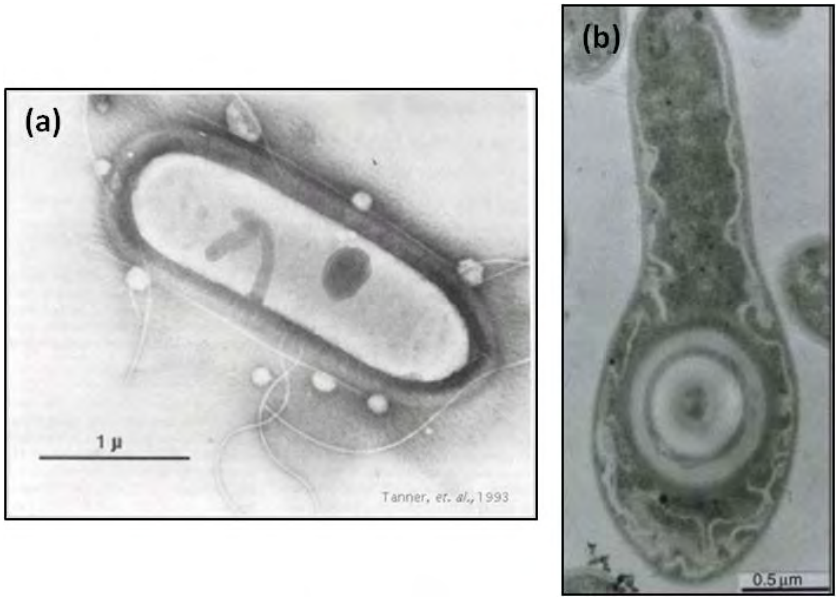


Figure 2.6: Microphotographs of two representatives of the carboxydotrophic acetogenic bacteria: (a) *Clostridium ljungdahlii* (Tanner et al., 1993); (b) *Moorella thermoacetica* (Drake et al., 2006). Bars: (a) 1 µm; (b) 0.5 µm.

ium ljungdahlii as well as *C. autoethanogenum* and *C. carboxidivorans* were extensively studied and are extensively used due to their ability to produce ethanol. *C. ljungdahlii* is able to ferment sugars, other organic compounds or CO_2/H_2 and syngas (CO/H_2). Due to the physiological characteristics of *C. ljungdahlii* and based on the information retrieved from its genome sequence, Köpke et al. (2010) referred that this organism can be used as a unique and novel biotechnological production platform based on syngas. To date, most of the known carboxydotrophic acetogens are mesophilic microorganisms, although there are also some important thermophilic acetogenic bacteria, namely *Moorella thermoacetica* and *Moorella thermoautotrophica*.

Moorella thermoacetica (f. *Clostridium thermoaceticum*) was isolated in 1942 (Collins et al., 1994; Fontaine et al., 1942), and is considered the model acetogen for most of the existing studies on the Wood-Ljungdahl pathway (Figure 2.6-b). The complete genome of *M. thermoacetica* was recently sequenced, in 2008, being the first acetogen to have a genome sequence published (Pierce et al., 2008). The genome sequence contributed not only to a further elucidation of the Wood-Ljungdahl pathway, but also helped to improve the understanding of the acetogenic metabolism and mode of energy conservation (Daniell et al., 2012; Pierce et al., 2008; Ragsdale and Pierce, 2008).

Acetogenic organisms have been intensively investigated for use in commercial syngas fermentation due to the high range of products formed: acetate, ethanol, butyrate, butanol and 2,3-butanediol (Köpke et al., 2011). Ethanol and butanol can be used as liquid fuels, replacing fossil fuels, while acetate and 2,3-butanediol are interesting building blocks for chemical industry

(Daniell et al., 2012).

2.2.2.3 Syngas/CO conversion by methanogenic archaea

Anaerobic CO utilization has been historically associated with acetogens but also with methanogens, that couple CO uptake to the production of acetate and methane, respectively. Methanogens are the best-studied archaea able to grow with CO as the sole energy source. Some mesophilic and thermophilic methanogenic archaea are able to directly reduce syngas and CO to methane (Tables 2.3, 2.4 and 2.5), namely *Methanosarcina barkeri*, *Methanosarcina acetivorans*, *Methanobrevibacter arboriphilicus* and *Methanothermobacter thermoautotrophicus*, according to the following reactions (Daniels et al., 1977; Henstra et al., 2007; O'Brien et al., 1984; Rother and Metcalf, 2004):

$4\,CO + 2\,H_2O \longrightarrow 3\,CO_2 + CH_4$

and

$CO + 3\,H_2 \longrightarrow CH_4 + H_2O$

On the other hand, CO can also be used by acetogenic or other bacteria able to perform the water shift reaction. The resulting acetate and H_2 could be further used by acetoclastic and hydrogenotrophic methanogens, respectively, following the reactions:

$CH_3COO^- + H_2O \longrightarrow CH_4 + HCO_3$

and

$4\,H_2 + HCO_3^- + H^+ \longrightarrow CH_4 + 3\,H_2O$

Moreover, hydrogenotrophic methanogens can utilize H_2 and CO_2 initially present in syngas for producing methane as well.

Methanogenic archaea conserve energy for growth by reducing some one- and two-carbon compounds to methane and generating, at the same time, an ion motive force over the cytoplasmic membrane for chemiosmotic ATP synthesis (Oelgeschläger and Rother, 2009).

M. acetivorans is the most well studied mesophilic methanogen with respect to the use of CO as growth substrate (Figure 2.7a) (Oelgeschläger and Rother, 2009; Rother and Metcalf, 2004; Rother et al., 2007). *Methanothermobacter thermoautotrophicus* was the first thermophilic methanogen described with the ability to grow on CO (Figure 2.7-b) (Sokolova et al., 2009; Zeikus and Wolee, 1972). *M. acetivorans* grows fast in the presence of high partial pressures of CO in comparison with *M. thermoautotrophicus* and *M. barkeri*. Interestingly, high partial pressures of CO inhibit methanogenesis in *M. acetivorans*, and this organism starts producing acetate and formate (Rother and Metcalf, 2004; Sipma et al., 2006). Rother and Metcalf (2004) showed that at CO partial pressure of approximately 0.8 bar, less than 5% of the metabolized CO was converted to methane. Methane production by *M. thermoautotrophicus* from CO is also affected by CO partial pressure above 0.3 bar (Daniels et al., 1977). Methanogens are generally more sensitive to elevated levels of CO than acetogens. The ability to metabolize CO without methane production, suggests an adaptation of carboxydotrophic methanogens to environments

with high CO concentrations.

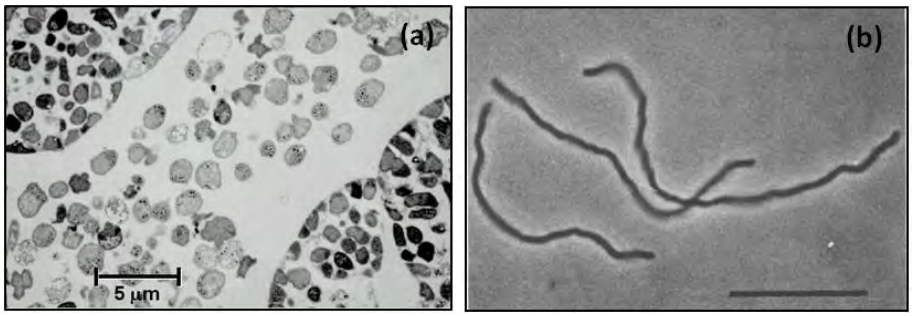


Figure 2.7: Microphotographs of two representatives of the carboxydotrophic methanogens: (a) *Methanosarcina acetivorans* (Galagan et al., 2002); (b) *Methanothermobacter thermoautotrophicus* (Zeikus and Wolee, 1972). Bars: (a) 5 µm; (b) 10 µm.

2.2.2.4 Syngas/CO conversion by hydrogenogens

Carboxydotrophic hydrogenogens are anaerobic microorganisms that can grow on CO and produce H_2 and CO_2 as sole products, according to the reaction $CO + H_2O \longrightarrow CO_2 + H_2$ (carboxydotrophic hydrogenogenesis) (Jiang et al., 2009; Sokolova et al., 2009)). The terms hydrogenogenic, hydrogenogens and hydrogenogenesis, were proposed by Svetlitchnyi et al. (2001) for the type of metabolism, the physiological group and H_2 formation process, respectively.

Anaerobic carboxydotrophic hydrogenogenic microorganisms conserve metabolic energy by oxidation of CO to CO_2 with the reduction of protons to molecular hydrogen (Henstra et al., 2007; Sipma et al., 2006). These reactions are catalyzed by two different enzymes: monofunctional CODH and Ech (Energy conserving hydrogenase), respectively, forming a membrane associated enzyme complex (Hedderich, 2004; Ragsdale, 2004). This complex facilitates CO oxidation, proton reduction and proton translocation. Ech plays a key role in energy generation in the carboxydotrophic hydrogenogenic metabolism, since this enzyme is responsible for H_2 production coupled with membrane translocation of protons, which generates a chemiosmotic gradient that can lead to ATP synthesis trough an ATP-synthase (Henstra et al., 2007; Sipma et al., 2006).

Over the past decade, the number of hydrogenogens described has rapidly increased, mainly found among thermophilic organisms (Sipma et al., 2006). The phylogenetic types differ, including representatives of bacteria and archaea. Most of the hydrogenogenic CO-oxidizing thermophiles were isolated from distinct natural hot environments with pH values from 5.5 to 10.0 and temperatures from 50 to 90 °C (Sokolova et al., 2009). The growth of these hydrogenogenic microorganisms is usually not supported by H_2/CO_2. Only few hydrogenogens are obligate carboxydotrophic; there are some that can also grow heterotrophically on other organic carbon compounds (Pusheva and Sokolova, 1995; Slepova et al., 2006; Sokolova et al., 2001, 2004, 2002;

Svetlichnyi et al., 1994).

Carboxydothermus hydrogenoformans was identifed as the first strict anaerobic thermophilic bacterium capable of CO oxidation and H_2 evolution (Figure 2.8-a) (Svetlichny et al., 1991). *C. hydrogenoformans* is one of the best-studied hydrogenogenic organisms for the following main reasons: i) it is an extreme thermophilic microorganism, growing at high temperature; ii) it could live on a diet of solely carbon monoxide; and iii) it converts water to hydrogen gas as part of its metabolism. The genome of *C. hydrogenoformans* has been sequenced and its analysis provided information about how this species is able to grow faster on CO than many other species, and presented also more insights into the metabolism of this organism (Wu et al., 2005). With all the information retrieved from the genome analysis of *Carboxydothermus hydrogenoformans* it became easier to use this microorganism as a source of biologically produced hydrogen gas (Wu et al., 2005).

Although the majority of known hydrogenogens is from the bacteria domain, the ability to grow at the expense of hydrogenogenic CO oxidation was also shown for some hyperthermophilic archaea of the genus *Thermococcus* (Kochetkova et al., 2011; Lee et al., 2012; Sokolova et al., 2004). *Thermococcus onnurineus* is a typical sulfur-reducing hyperthermophilic archaeon and was shown to use CO as a carbon and energy source and converting it to CO_2 and H_2 (Figure 2.8-b) (Lee et al., 2012). Genome sequence analysis confirmed that *T. onnurineus* has all the metabolic pathways necessary for carboxydotrophic growth (Lee et al., 2008). Hydrogenogens can be used for biological production of hydrogen gas, which make this type of microorganisms very attractive for biotechnological applications.

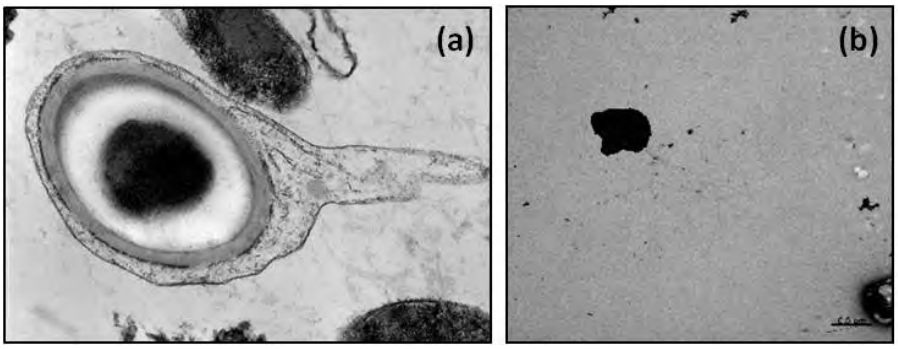


Figure 2.8: Microphotographs of two representatives of the carboxydotrophic hydrogenogens. (a) *Carboxydothermus hydrogenoformans* (Wu et al., 2005); (b) *Thermococcus onnurineus* (Seob et al., 2006). Bar: (b) 0.5 µm.

2.2.2.5 Syngas/CO conversion by sulfate reducing prokaryotes

Sulfate reducers are anaerobic microorganisms that are able to use sulfate as a terminal electron acceptor. Some sulfate reducers are able to utilize CO as energy source according to the follow

reaction:

$$4\,CO + SO_4^{2-} + H^+ \longrightarrow 4\,CO_2 + HS^-$$

Although sulfate reducing prokaryotes (SRP) are usually inhibited by CO, most SRP are reported to utilize CO as an energy source, convert it CO to CO_2 and H_2 and subsequently use the H_2 for sulfate reduction (Oelgeschläger and Rother, 2008). However, some other SRP, such as *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Figure 2.9-a) or *D. kuznetsovii*, do not only generate H_2, but also produce acetate when growing with CO (Parshina et al., 2005).

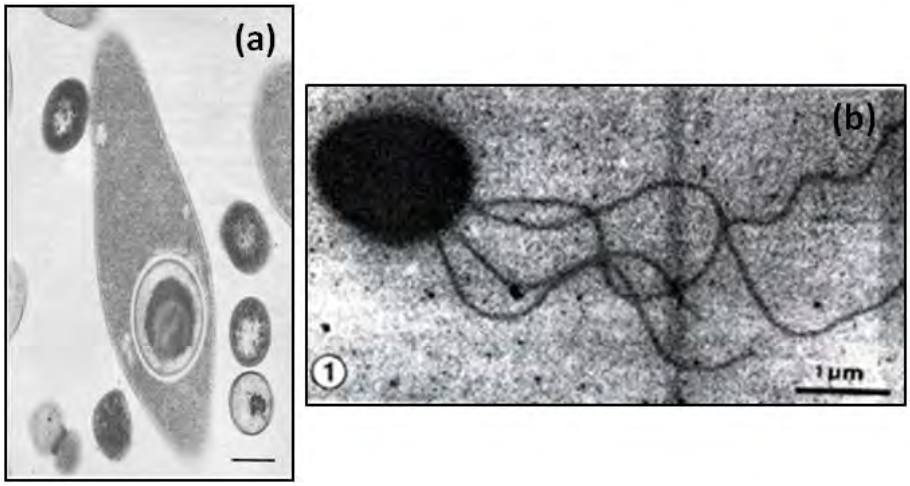


Figure 2.9: Microphotographs of two representatives of the carboxydotrophic sulfate reducers. (a) *Desulfotomaculum thermobenzoicum* subsp. *thermosyntrophicum* (Plugge et al., 2002); (b) *Archaeoglobus fulgidus* (Stetter, 1988). Bars: (a) 0.5 µm; (b) 1 µm.

It is also known that CO is better tolerated by thermophilic sulfate reducers, when compared with mesophilic SRP (Parshina et al., 2005). There are several SRP able to convert CO at concentrations up to 20%, but higher concentrations completely inhibited growth. Parshina et al. (2005) also emphasize that if sulfate reducers are co-cultivated with carboxydotrophic bacteria high CO concentrations are tolerated.

The most studied CO utilizing SRP are species of the genera *Desulfovibrio* and *Desulfotomaculum* (Parshina et al., 2010). *Desulfovibrio vulgaris* was the first sulfate reducer described to be capable of use CO for sulfate reduction, converting CO into H_2 and CO_2, and using H_2 for sulfate reduction (Lupton et al., 1984). Another very interesting example of a sulfate reducing bacterium due to its ability to grow in the presence of 100% CO is *Desulfotomaculum carboxydivorans*, that is also capable of growing in the absence of sulfate, as a hydrogenogen (Parshina et al., 2005).

It was also shown that the anaerobic extremely thermophilic *Archaeoglobus fulgidus*, a sulfate-reducing archaeon, is capable of autotrophic growth with CO (Figure 2.9-b). Oxidation of CO to CO_2 was coupled to sulfate reduction. In the absence of sulfate, it was reported that the only

products of CO metabolism were acetate (formed via the reductive acetyl-CoA pathway) and formate (Henstra et al., 2007; Sokolova et al., 2009). Sulfate reduction to sulfide is not inhibited by CO (Parshina et al., 2010).

The existence of CO tolerant and CO oxidizing sulfate reducers is a new discovery that opens perspectives for their biotechonological utilization, such as for the possibility of using CO-rich syngas as a cheap alternative electron donor for biodesulfurization processes (Parshina et al., 2010; Sipma et al., 2006).

2.3 Biotechnological applications of CO converting microorganisms

Since the last few years advances were made with the study of microbial CO metabolism aiming to assess the feasibility of a biotechnological process that could be an alternative for the biofuels and chemicals production.

Due to the fast and increase progress of synthetic biology and metabolic engineering, the information available from the genome sequences of the carboxydotrophic microorganisms offers significant potential for syngas fermentation process. Several biochemical reactions of the reductive acetyl-CoA pathway were elucidated when using the genome sequences from the selected model organisms, namely, *Moorella thermoacetica*, *Clostridium ljungdahlii*, *Clostridium carboxidivorans* and *Eubacterium limosum*.

Nowadays, most of the biotechnological interest is on the acetogenic bacteria that have the ability to convert CO into ethanol, via acetyl-CoA pathway. Ethanol is the biofuel produced in higher amounts worldwide. Gasification process and further syngas conversion to ethanol has been shown to be commercially feasible. *Clostridium ljungdahlii* is an example of a well studied microorganism that is used to ferment syngas to ethanol as a commercial process (Köpke et al., 2010). Most other studies on syngas anaerobic conversion have focused on the utilization of pure cultures of microorganisms as a biotechnological tool for ethanol production: *Alkalibaculum bacchi*, *Butyribacterium methylotrophicum*, *Clostridium autoethanogenum*, *C. carboxidivorans*, *C. drakei* and *C. ragsdalei* (Abrini et al., 1994; Allen et al., 2010; Daniell et al., 2012; Köpke et al., 2011; Kundiyana et al., 2011; Lynd et al., 1982).

Production of butanol by carboxydotrophic organisms is also subject of study due to its potential biotechnological application. Butanol is considered an extraordinary alternative fuel, since its energy content is 30% higher than ethanol (Tirado-Acevedo et al., 2010). Butanol production by *Butyribacterium methylotrophicum* from syngas was already very well studied. This organism is one of the main versatile CO-utilizing bacteria, since it is able to produce not only butanol, but also ethanol, acetate and butyrate (Henstra et al., 2007; Lynd et al., 1982).

Very recently, the biotechnological interest on the *Clostridium autoethanogenum*, *C. ljungdahlii*, and *C. ragsdalei* was increased since these organisms are able to produce 2,3-butanediol

using a CO-containing industrial waste gas or syngas as the sole energy and carbon source (Köpke et al., 2011). 2,3-butanediol is considered a high-value chemical, since it is a precursor in the manufacture of a variety of chemical products.

Another discovery that increases the biotechnological interest on syngas utilization is the ability of anaerobic hydrogenogenic microorganisms of removing CO coupled with H_2 production. This is a biological alternative for the chemical water-gas-shift reaction. Increased attention has been given to thermophilic carboxydotrophic hydrogenogenic bacteria, e.g. *Carboxydothermus hydrogenoformans*, *Carboxydocella thermoautotrophica*, *Thermincola carboxydiphila* and *Desulfotomaculum carboxydivorans* that convert CO and H_2O to H_2 and CO_2 (Henstra et al., 2007; Sokolova et al., 2009). Furthermore, utilization of CO containing syngas for biological desulfurization is an important application. This is possible due to the existence of carboxydotrophic sulfate reducing bacteria microorganisms that can perform a high-rate hydrogenogenic CO conversion coupled with sulfate reduction (Parshina et al., 2010; Sipma et al., 2006).

As stated by Sipma et al. (2006) there are some other interesting biotechnological applications whereas no growing cultures of carboxydotrophic organisms are used, which can rely on the utilization of the purified enzymes from these microorganisms. As some examples, purified CODH can be used: in biofilters for cleaning the air; can be applied in dechlorination process; or in the reductive carboxylation of phenols.

2.4 Conclusions and perspectives

Syngas fermentation to biofuels and chemicals is an attractive technology and it is a sustainable alternative for the fast depleting fossil fuels. In the past years, research has been done in the area of syngas fermentation, with the isolation of new syngas fermenting organisms, and the development of bioreactors for syngas microbial conversion. Syngas- converting microorganisms utilize the Wood-Ljungdahl pathway to produce fuels and valuable chemicals including acetate, ethanol, butanol and 2,3-butanediol. Research efforts should also be directed to the production of gaseous biofuels, such as methane or hydrogen. The commercialization of syngas fermentation to biofuels is not yet efficient because of the gas-to-liquid mass transfer limitations and low product yield. One of the main challenges in designing bioreactors for commercial conversion of syngas is to significantly improve mass transfer from the gaseous substrates into the aqueous phase and, consequently, to the microorganisms that are in the liquid phase. Another aspect that could be optimized regards to the product yield and, even though known syngas-fermenting microbes show high specificity, their product yields are still low. For that reason, the isolation of novel syngas/CO-rich gas degrading bacteria with high product yield is necessary for successful commercialization of syngas fermentation technology. Even though a number of microorganisms have been isolated with the abilities to convert syngas and CO to biofuels the list is still small and physiological and metabolic research for most of these isolates is poor. Another important

aspect to study is the possibility for bacteria improvement by modifying the available syngas fermenting bacteria with genetic engineering techniques. The combination of new and better isolates with advancement in metabolic engineering would improve gasification-fermentation process cost, product yields and overall performance.

Chapter 3

Enrichment of anaerobic syngas converting bacteria from thermophilic bioreactor sludge

Thermophilic 55 °C anaerobic microbial communities were enriched with a synthetic syngas mixture (composed of CO, H_2 and CO_2) or with CO alone. Cultures T-Syn and T-CO were incubated and successively transferred with syngas (16 transfers) or CO (9 transfers), respectively, with increasing CO partial pressures from 0.09 to 0.88 bar. Culture T-Syn, after 4 successive transfers with syngas, was also incubated with CO and subsequently transferred (9 transfers) with solely this substrate - cultures T-Syn-CO. Incubation with syngas and CO caused a rapid decrease in the microbial diversity of the anaerobic consortium. T-Syn and T-Syn-CO showed identical microbial composition, and were dominated by *Desulfotomaculum* and *Caloribacterium* species. Incubation initiated with CO resulted in the enrichment of bacteria from the genera *Thermincola* and *Thermoanaerobacter*. Methane was detected in the first two to three transfers of T-Syn, but production ceased afterwards. Acetate was the main product formed by T-Syn and T-Syn-CO. Enriched T-CO cultures showed a two-phase conversion, in which H_2 was formed first and then converted to acetate. This research provides insight into how thermophilic anaerobic communities develop using syngas/CO as sole energy and carbon source can be steered for specific end products and subsequent microbial synthesis of chemicals.

3.1 Introduction

Syn(thesis)gas is a gaseous mixture mainly composed by carbon monoxide, hydrogen and carbon dioxide. Syngas is produced during the gasification of carbon-containing materials and its further conversion to bulk chemicals and fuels, using chemical or biotechnological processes, can be a way of recycling lignocellulosic biomass or even recalcitrant wastes (Sipma et al., 2006). One of the major problems of using chemical catalytic processes for syngas conversion (to *e.g.* methane, organic acids and alcohols, or hydrocarbons) is the requirement of a constant CO/H_2 ratio, worsened by the fact that CO is poisoning to most metal catalysts. Gasification process conditions and feedstocks greatly influence syngas composition – the utilization of microbial catalysts, which are less affected by oscillations in syngas composition and can tolerate the presence of trace contaminants, needs to be explored for the development of novel biotechnological processes for syngas valorization (Heiskanen et al., 2007; Henstra et al., 2007; Tirado-Acevedo et al., 2010).

Carbon monoxide is a direct substrate for a variety of anaerobic microorganisms that can produce different added-value products, such as hydrogen, methane, fatty-acids, and alcohols (for complete review see Bruant et al. (2010); Guiot et al. (2011); Henstra et al. (2007); Köpke et al. (2011). Carboxydotrophic hydrogenogenic bacteria, e.g. *Rhodospirillum rubrum*, *Carboxydothermus hydrogenoformans*, *Carboxydocella thermoautotrophica*, *Thermincola carboxydiphila* and *Desulfotomaculum carboxydivorans* can convert CO and water to H_2 and CO_2. *Clostridium* species are able to produce acetate and alcohols from CO (Henstra et al., 2007), while *Moorella* species generally produce acetate or hydrogen (Alves et al., 2013; Sokolova et al., 2009). Methane can be directly produced from CO by methanogenic archaea, such as *Methanosarcina* species and the thermophile *Methanothermobacter thermoautotrophicus* (Daniels et al., 1977; Rother and Metcalf, 2004). Using a mixed culture approach, the possible routes for CO /syngas conversion increase, as well as the robustness of the microbial system. Providing different environmental conditions to open mixed cultures could possible deviate CO/syngas conversion in different routes. Nevertheless, mixed culture approaches for the conversion of these substrates have received little attention. Sipma et al. (2003, 2004) reported CO conversion by six different mesophilic anaerobic bioreactor sludges. Sludges incubated at 30 °C produced methane and/or acetate, while incubation at 55 °C resulted in the formation of mainly methane. Interestingly, CO conversion at thermophilic conditions was significantly faster, despite the fact that the sludges were not acclimated to high temperature. Thermophilic conditions triggered a change in the metabolic route for CO conversion; at 30 °C conversion of CO to methane was dependent on homoacetogenic CO-degraders and acetoclastic methanogens, while at 55 °C hydrogenogenic CO-degraders and hydrogenotrophic methanogens were the main players. Guiot et al. (2011) also observed a higher CO-conversion potential during batch incubation of anaerobic granular sludge under thermophilic conditions (5-fold higher than under mesophilic conditions). However,

in these studies, the effect of long term exposure to CO was not studied, neither the microbial composition of the sludges was analyzed. In the present work we intend to get insight on the effect of long term exposure of a thermophilic anaerobic sludge to syngas and CO, by studying both microbial physiology aspects and community composition after subsequent culture transfer in the presence of syngas/CO.

3.2 Materials and Methods

3.2.1 Source of inoculum

Anaerobic suspended sludge, obtained from an anaerobic reactor treating the organic fraction of municipal solid wastes (Barcelona, Spain), was used as seed sludge for starting the enrichment series under thermophilic conditions (55 °C).

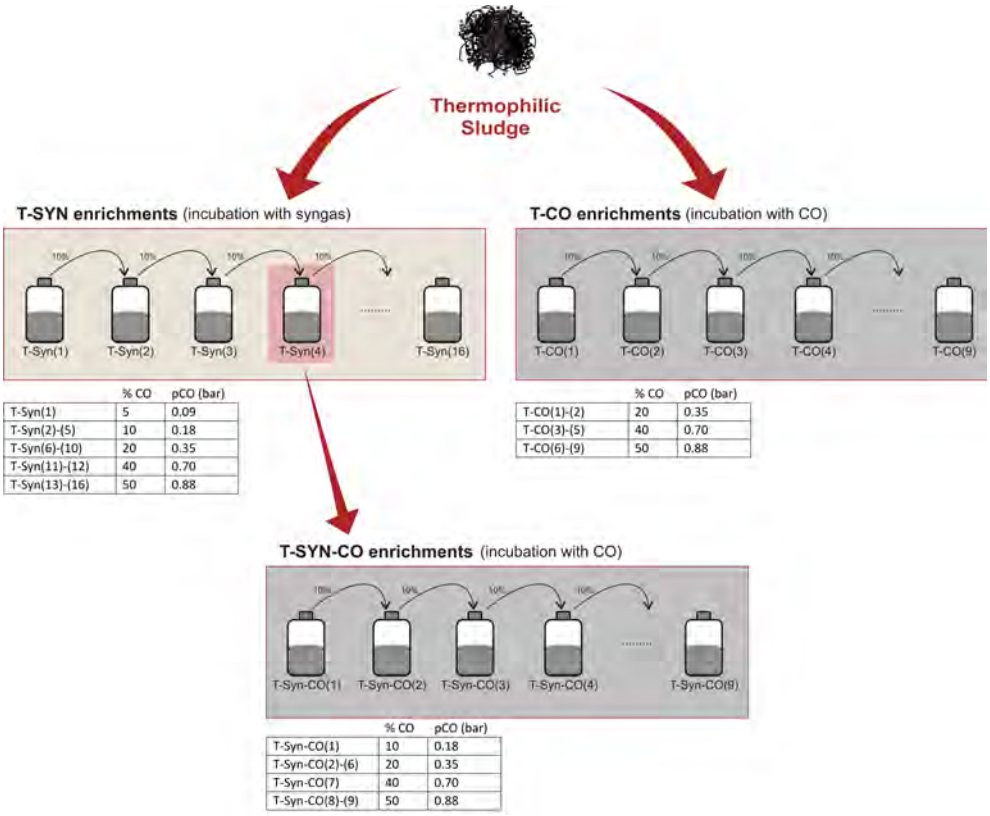
3.2.2 Medium composition

A phosphate-buffered mineral salt medium (20 mM, pH 7.0) was used for cultivation of the enrichment cultures. The medium contained the following (grams per liter): Na_2HPO_4, 1.63; NaH_2PO_4, 1.02; NH_4Cl, 0.3; $CaCl_2 \cdot 2\,H_2O$, 0.11; $MgCl_2 \cdot 6\,H_2O$, 0.10; NaCl, 0.3; resazurin, 0.05. Acid and alkaline trace elements solutions (1 mL per liter of each) and vitamins solution (0.2 mL per liter) were also supplemented to the medium (Stams et al., 1993). The mineral medium was dispensed into serum bottles, sealed with butyl rubber septa and aluminum crimp caps and flushed with a mixture of H_2/CO_2 (80:20 vol/vol) or with N_2. Synthetic syngas mixture (60% CO, 30% H_2, 10% CO_2) or pure CO were added to the bottles' headspace to the desired final partial pressure using a syringe. Final total gas pressure in the bottles' headspace was 1.75 bar. The bottles were autoclaved for 20 min at 121 °C. Before inoculation, mineral medium was reduced with sodium sulfide to a final concentration of 2 mM. All the inoculations and transfers, as well as addition of stock solutions, were performed aseptically using sterile syringes and needles.

3.2.3 Enrichment cultures

Thermophilic anaerobic sludge was used for the startup of two syngas- and CO-converting enrichment series: T-Syn and T-CO ("T" stands for thermophilic (55 °C) enrichments; "Syn" - syngas and "CO" - carbon monoxide) (see Figure 3.1 for a schematic representation of the experimental setup). Later, a T-Syn-CO enrichment series, was started by incubating enrichment culture T-Syn(4) with CO (the number in between parenthesis refers to the number of transfer). Enrichment cultures were developed by successive transfers of active cultures (10% v/v) into fresh medium with the headspace filled with increasing concentrations of CO (from 5% (pCO = 0.09 bar) to 50% (pCO = 0.88 bar)). Syngas was diluted with H_2/CO_2, and pure CO diluted

with N_2, in order to get the desired final CO partial pressure (total pressure was kept constant at 1.75 bar). The enrichments were done in duplicate. Bottles were incubated with agitation (100 rpm) and in the dark.



	% CO	pCO (bar)
T-Syn(1)	5	0.09
T-Syn(2)-(5)	10	0.18
T-Syn(6)-(10)	20	0.35
T-Syn(11)-(12)	40	0.70
T-Syn(13)-(16)	50	0.88

	% CO	pCO (bar)
T-CO(1)-(2)	20	0.35
T-CO(3)-(5)	40	0.70
T-CO(6)-(9)	50	0.88

	% CO	pCO (bar)
T-Syn-CO(1)	10	0.18
T-Syn-CO(2)-(6)	20	0.35
T-Syn-CO(7)	40	0.70
T-Syn-CO(8)-(9)	50	0.88

Figure 3.1: Flowsheet diagram of the experimental procedure used for obtaining syngas/CO thermophilic enrichments.

3.2.4 Analytical methods

Gas samples were analyzed by GC with a GC-Chrompack 9001 with a thermal conductivity detector and equipped with two columns: a Porapack Q ($100-180$ mesh) $2\,\text{m} \times 1/8" \times 2.0\,\text{mm}$ SS column, and a MolSieve 5A ($80-100$ mesh) $1.0\,\text{m} \times 1/8" \times 2.0\,\text{mm}$ SS column. Argon was the carrier gas at a flow rate of $16\,\text{mL}\,\text{min}^{-1}$. The oven, injector and detector temperatures were 35 °C, 110 °C and 110 °C, respectively. Volatile fatty acids (VFA), ethanol and butanol were determined by high performance liquid chromatography using an HPLC (Jasco, Tokyo, Japan) with a Chrompack column (6.5×30 mm^2), coupled to a UV detector at 210 nm and a RI detector. The mobile phase used was sulfuric acid (0.01 N) at a flow rate of $0.6\,\text{mL}\,\text{min}^{-1}$. Column temperature was set at 60 °C.

3.2.5 DNA extraction and amplification

20 mL aliquots of well-homogenized microbial cultures were concentrated by centrifugation (13400 g, 15 min), immediately frozen and stored at −20 °C. Total genomic DNA was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA) in accordance with the manufacturer's instructions. Bacterial and archaeal 16S rRNA genes were amplified by PCR using a Taq DNA polymerase kit (Invitrogen, Carlsbad, CA, USA); reaction mixtures and PCR programs used were as described elsewhere (Sousa et al., 2009). Primer sets U968-f/L1401-r and Bact27f/Uni1492r were used for 16S rRNA gene amplification for denaturing gradient gel electrophoresis (DGGE) and sequencing purposes, respectively (Lane, 1991; Nübel et al., 1996). Primer set arch109f/Uni1492r was used for archaeal 16S rRNA gene amplification (Lane, 1991; Großkopf et al., 1998). A 40 bp GC-clamp was added at the 5' end sequence of the primer U968f (Muyzer et al., 1993). Size and yield of PCR products were estimated by electrophoresis in a 1% agarose gel (wt/vol), using a 100 bp BLUE eXtended DNA Ladder (BIORON, Ludwigshafen, Germany) and ethidium bromide staining.

3.2.6 DGGE analysis

DGGE analysis of the PCR products was performed by using the DCode system (Bio-Rad, Hercules, CA, USA). Gels containing 8% (wt/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) were used with a linear denaturing gradient of 30 − 60%, with 100% of denaturant corresponding to 7 M urea and 40% (vol/vol) formamide. Electrophoresis was performed for 16 h at 85 V and 60 °C in a 0.5× Tris-Acetate-EDTA buffer. DGGE gels were stained with silver nitrate (Sanguinetti et al., 1994) and scanned in an Epson Perfection V750 PRO (Epson, USA).

DGGE gels were scanned at 400 dpi and analyzed using the BioNumericsTM software package (version 5.0; Applied Maths BVBA, Sint-Martens-Latem, Belgium). The normalized banding patterns were used to generate dendrograms by calculating the Pearson's product moment correlation coefficient (Cole et al., 2003). The unweighted pair group method with arithmetic averages (UPGMA) was further applied for clustering.

3.2.7 Cloning and sequencing

PCR products obtained from the enrichment samples genomic DNA were purified using the PCR Clean Up kit NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). After purification, PCR amplicons were ligated into the pGEM-T vector using the pGEM Easy Vector Systems (Promega, Madison, WI, USA), and introduced into competent *Escherichia coli* 10G (Lucigen Corporation, Middleton, MI, USA), according to the manufacturer's instructions. Positive transformants were selected (by blue/white screening) and grown in appropriate media supplemented with ampicillin. Insert size was confirmed by PCR amplification with the pGEM-T-specific primers PG1-f and PG2-r to confirm the size of the inserts. To assign the composition

of the predominant community visualized in the DGGE-patterns, nearly full-length bacterial 16S rRNA gene fragments, retrieved from enrichment cultures, were used to construct clone libraries. Amplified ribosomal DNA restriction analysis (ARDRA) was used to screen clone libraries for redundancy as described elsewhere (Sousa et al., 2009, 2007). Clones with the same electrophoretic mobility as that of predominant bands of DGGE-patterns were purified using the Nucleo Spin Extract II purification kit and subjected to nucleotide sequence analysis. Sequencing reactions were performed at Biopremier (Lisbon, Portugal) using pGEM-T vector-targeted sequencing primers SP6 and T7 and internal specifically tailored primers, when needed. Partial sequences were assembled by using the Contig Assembly Program (CAP) application included in the BioEdit v7.0.9 software package (Hall, 1999; Huang, 1992). Consensus sequences obtained were checked for potential chimera artifacts using Bellerophon software (Huber et al., 2004).

3.2.8 Phylogenetic analysis and nucleotide sequence accession numbers

Similarity searches for the 16S rRNA gene sequences derived from the clones were performed using the NCBI BLAST search program within the GenBank database[1] (Altschul et al., 1990). Nucleotide sequences obtained in this study have been deposited in the European Nucleotide Archive (ENA) under accession numbers HF562211 to HF562214.

3.3 Results

3.3.1 Syngas and CO conversion by thermophilic enrichments

T-Syn cultures were initially incubated with low CO-content syngas (5%, pCO = 0.09 bar) and by the third transfer CO concentration was doubled (pCO = 0.18 bar). Carbon monoxide and H_2 were converted to methane by the initial T-Syn enrichments (Table 3.1). After 3 transfers, T-Syn(3) cultures were able to convert about 82% of the initially supplied CO to methane. However, in T-Syn(4) acetate was the main product detected from CO conversion and methane was no longer produced. T-CO cultures, which were initially incubated with 20% CO (pCO = 0.35 bar), and T-Syn-CO cultures (deriving from T-Syn(4)), did not produce methane during the entire experiment. Acetate was the main product formed in T-Syn-CO(1), while T-CO (1) converted CO to mainly hydrogen (data not shown). 16S rRNA gene archaeal-specific PCR indicated the presence of methanogens in the inoculum sample and in the T-Syn samples corresponding to the first 3 initial transfers (T-Syn(1) to T-Syn(3)); all the other enrichments were negative for the presence of archaeal phylotypes (data not shown).

T-Syn, T-CO and T-Syn-CO were successfully sub-cultured for over one year (T-Syn, 16 transfers with syngas; T-CO, 9 transfers with CO; and, T-Syn-CO, 4 transfers with syngas followed by 9 transfers with CO), leading to microbial enrichments with stable physiological

[1] http://www.ncbi.nlm.nih.gov/blast/

Table 3.1: Syngas conversion by initial thermophilic (T-Syn(x)) enrichment cultures (Ptotal = 1.75 bar); x represents the number of successive transfers.

	T-Syn (initial transfers)		
	T-Syn(1) (5% CO)	T-Syn(3) (10% CO)	T-Syn(4) (10% CO)
Incubation time (days)	14	33	27
Substrate composition			
Carbon monoxide ($\mathrm{mmol\,L^{-1}_{medium}}$)	2 ± 1	6 ± 0	5
Carbon dioxide ($\mathrm{mmol\,L^{-1}_{medium}}$)	28 ± 1	17 ± 1	21
Hydrogen ($\mathrm{mmol\,L^{-1}_{medium}}$)	93 ± 3	92 ± 0	83
Substrate utilization			
Carbon monoxide (%)	100 ± 0	82 ± 4	100
Hydrogen (%)	100 ± 0	100 ± 0	0
Products formed			
Acetate (mM)	0	0.8	2.1
Methane ($\mathrm{mmol\,L^{-1}}$)	22 ± 2	20 ± 1	0

* Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using the Henry law. Concentration of gases is expressed as mmol per liter of medium.

properties. Highly enriched T-Syn and T-Syn-CO cultures produced mainly acetate from syngas or CO (Figure 3.2a and 3.2b), while in T-CO enriched cultures hydrogen was primarily formed and only further converted to acetate (Figure 3.2c).

In T-Syn cultures (Figure 3.2a) there was a slight increase on H_2 concentration while CO decreased, suggesting that about 3 $mmolL^{-1}_{medium}$ of CO were converted to H_2, likely according to equation 1. The remaining CO (16.1± 2.6 $mmolL^{-1}_{medium}$) was converted to acetate, which was associated with H_2 consumption. This could result from the combination of distinct acetate-producing routes, i.e. directly from CO (eq. C 3.2) or from the reaction between CO with hydrogen (eq. C 3.3). Additional acetate is likely being produced from H_2/CO_2 (eq. C 3.4) because, even after complete CO depletion, CO_2 and H_2 decrease steadily. At the end of the incubation, (9.4± 1.1 mM acetate had accumulated, which accounts for approximately 97% of product recovery (considering maximum theoretical acetate production from the consumed CO and H_2). Propionate was the only other organic compound produced (maximum 1.0± 0.1 mM); methane or alcohols were not detected in the T-Syn incubation.

$$CO + H_2O \longrightarrow CO_2 + H_2 \quad \text{(C 3.1)}$$

$$4\,CO + 2\,H_2O \longrightarrow CH_3COOH + 2\,CO_2 \quad \text{(C 3.2)}$$

$$2\,CO + 2\,H_2 \longrightarrow CH_3COOH \quad \text{(C 3.3)}$$

$$4\,H_2 + 2\,CO_2 \longrightarrow CH_3COOH + 2\,H_2O \quad \text{(C 3.4)}$$

Acetogenic activity from CO/H_2 (eq. C 3.3) seems also to be predominant in cultures T-Syn-CO; in these cultures 25.6± 2.3 $mmolL^{-1}_{medium}$ CO were converted to 7± 1 mM acetate and 12± 0.6 $mmolL^{-1}_{medium}$ CO_2 (Figure 3.2b). Conversely, in cultures T-CO, an intermediary formation of hydrogen points to a hydrogenogenic route for CO conversion (eq. C 3.1); by day 16 of incubation, 10 $mmolL^{-1}_{medium}$ H_2 have been produced from CO in T-CO cultures (Figure 3.2c). However, extension of the incubation time led to the consumption of H_2/CO_2, with the formation of 2.4 mM acetate (eq. C 3.4).

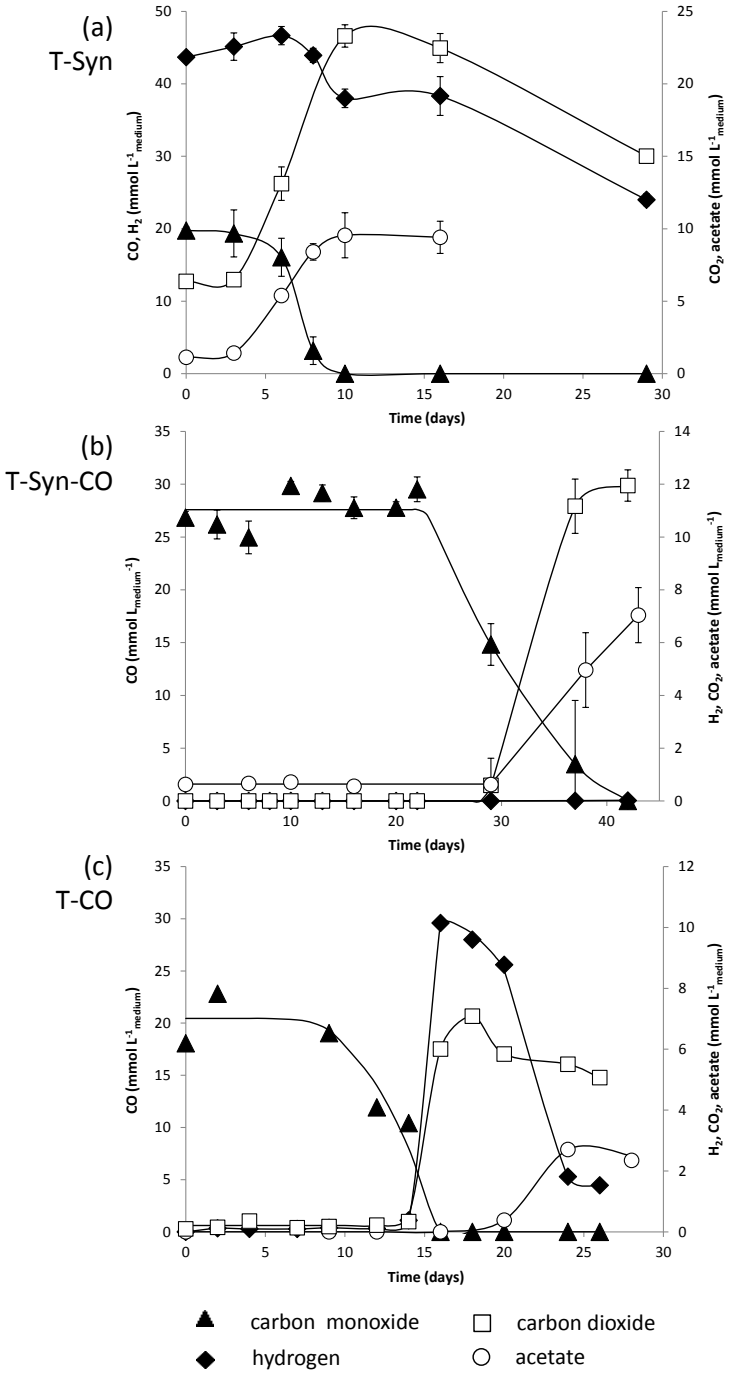


Figure 3.2: Substrate consumption and product formation by enriched syngas- and CO-degrading cultures. (a) T-Syn, (b) T-Syn-CO, and (c) T-CO. Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using the Henry law. Gas products values are expressed in relation to volume of medium, i.e. mmol of gas measured in the headspace divided by the volume of liquid medium.

3.3.2 Molecular characterization of the thermophilic enrichments

Microbial communities present in the thermophilic enrichment series were analyzed by DGGE fingerprinting of PCR-amplified 16S rRNA gene fragments (Figure 3.3).

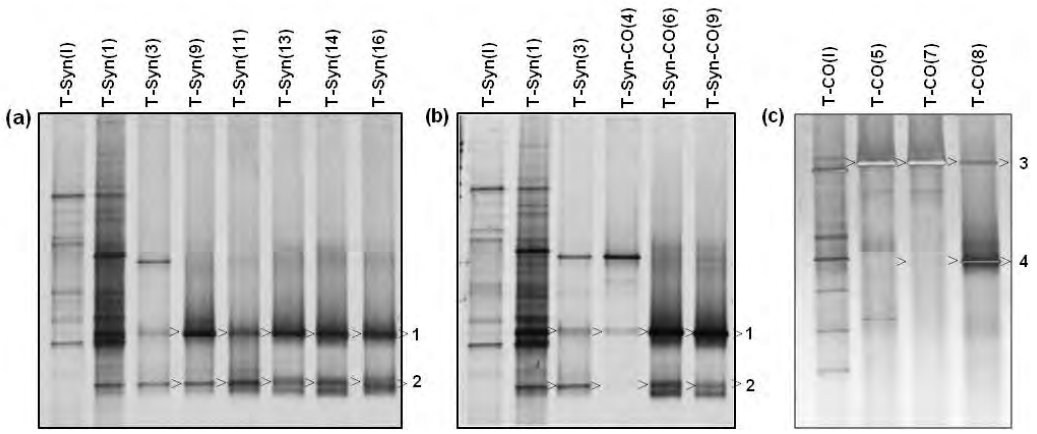


Figure 3.3: Bacterial DGGE profile of the thermophilic enrichment series (a) T-Syn, (b) T-Syn-CO and (c) T-CO. I, inoculum; T-Syn(x), T-Syn-CO(x) and T-CO(x), enrichment cultures, where x represents the number of successive transfers at sampling point.

The decrease in the number of predominant bands, from the inoculum sample profile to subsequent transfers, indicates a rapid reduction in bacterial richness. Similarity coefficients of 52, 33 and 35% between the inoculum and the samples T-Syn(9), T-Syn-CO(4) and T-CO(5), respectively, were obtained. DGGE profiles from T-Syn(9) and T-Syn-CO(4) transfers onwards, showed to be stable, with similarity coefficients between 90% and 97%, for both cultures. Moreover, T-Syn and T-Syn-CO enrichments showed a similar stable DGGE profile, in which only two bands are predominant - bands 1 and 2 (Figure 3.3a and 3.3b). DGGE profile obtained for T-CO was clearly different (Figure 3.3c). DGGE profile of T-CO had also two predominant bands - bands 3 and 4 (Figure 3.4). During the first period of conversion, corresponding to CO conversion to H_2, band 3 is the predominant band (Figure 3.4, sampling point T-CO(9)-1). With the extension of incubation time, and further H_2 depletion, band 4 became also predominant (Figure 3.4, sampling point T-CO(9)-2).

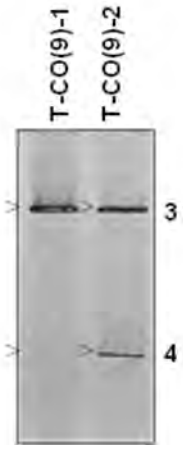
Bands 1 to 4 were identified by 16S rRNA gene sequencing (Table 3.2). Predominant ribotypes in T-Syn and T-Syn-CO (bands 1 and 2) were most closely affiliated with the genera *Desulfotomaculum* (*D. australicum* 98% 16S rRNA gene identity) and *Caloribacterium* (*C. cisternae* 94% 16S rRNA gene identity). Culture T-CO was composed also of two predominant microorganisms (bands 3 and 4), clustering with the genera *Thermincola* (*T. carboxydiphila*, 99% 16S rRNA gene identity) and *Thermoanaerobacter* (*T. thermohydrosulfuricus*, 97% 16S rRNA gene identity).

Table 3.2: Phylogenetic affiliations of cloned 16S rRNA gene sequences corresponding to the identified bands in DGGE profiles.

Band ID	Phylum[a]	Class[a]	Relative abundance[b]	Closest relatives	Identity	Accession no.
1	Firmicutes	Clostridia	67.5%	*Desulfotomaculum* sp. Hbr7	99%	HF562211
				Desulfotomaculum australicum strain AB33	98%	
2	Firmicutes	Clostridia	27.9%	Thermophilic anaerobic bacterium K1L1	96%	HF562212
				Caloribacterium cisternae SGL43^{T}	94%	
3	Firmicutes	Clostridia	79.2%	*Thermincola* sp. JR	99%	HF562213
				Thermincola carboxydiphila strain 2204	98%	
4	Firmicutes	Clostridia	7.2%	*Thermoanaerobacter thermohydrosulfuricus* strain JCM 9674	97%	HF562214

[a] Classified using the RDP Naïve Bayesian Classifier.

[b] Relative abundance of clones with identical ARDRA profiles (calculated from a total of 74 and 69 clones retrieved from T-Syn/T-Syn-CO and T-CO cultures, respectively).



	Incubation time (days)	H2 (mmol L^{-1})
T-CO(9)-1	21	8.4
T-CO(9)-2	42	0.2

Figure 3.4: Bacterial DGGE profile of T-CO enrichment in two sampling points during 9^{th} transfer (T-CO(9)). T-CO(9)-1: early incubation; hydrogen production. T-CO(9)-2: later incubation; hydrogen depletion.

3.4 Discussion

Using thermophilic methanogenic sludge as inoculum, syngas/CO converting thermophilic communities were obtained after an enrichment period of over one year. The microbial diversity of the cultures declined rapidly upon subculturing. Methanogenic activity of the sludge was rapidly affected by CO. Methane was not produced when the anaerobic sludge was incubated with CO alone (culture T-CO) and, in the incubations with syngas, methane production ceased after 4 subsequent transfers. Although there are some methanogenic archaea that are able to convert CO to methane (e.g. *Methanosarcina barkeri*, *Methanosarcina acetivorans* and *Methanothermobacter thermoautotrophicus*) most methanogens do not tolerate CO (Guiot et al., 2011; Henstra et al., 2007; Rother and Metcalf, 2004). Moreover, acetogenic and hydrogenogenic CO-converting microorganisms grow faster than methanogens (Sipma et al., 2006). Carbon monoxide partial pressure might also influence microbial dynamics; the faster inhibition of the methanogenic activity in T-CO might be related to the higher CO partial pressure applied in the beginning of the enrichment series, when compared to T-Syn (pCO of 0.35 bar and 0.09 bar for T-CO(1) and T-Syn(1), respectively). Nevertheless, one cannot disregard the presence of H_2 in the syngas mixture that is a direct substrate of hydrogenotrophic methanogens; methanogenic activity in syngas initial enrichments might partially rely on the H_2 present in the substrate. Guiot et al. (2011) reported a complete inhibition of methanogenesis in anaerobic sludge subjected to CO partial pressure between 0.30 and 0.83 bar. Also, methane production by *M. thermoautotrophicus* from CO is inhibited by a CO partial pressure above 0.3 bar (Daniels et al., 1977). Acetogenic and hydrogenogenic bacteria seem to be more tolerant to CO. For instance, growth of the acetogen *Blautia producta* is stimulated with increasing CO up to a value of CO par-

tial pressure of 0.8 bar and lag phases appear only at CO partial pressure of 1.5 bar and above (Vega et al., 1989). Also, no lag-phase was observed on H_2 production by *Desulfotomaculum carboxydivorans* with 1.8 bar CO (without sulfate) (Parshina et al., 2005).

Enriched cultures T-Syn and T-Syn-CO converted CO to mainly acetate, while cultures T-CO showed a two-phase profile - H_2 was produced from CO and subsequently converted to acetate. DGGE analysis of the predominant bacterial populations present in the enriched cultures revealed phylotypes affiliated with only a few genera: *Desulfotomaculum* and *Caloribacterium* species were predominant in T-Syn and T-Syn-CO, while *Thermincola* and *Thermoanaerobacter* related bacteria were predominant in T-CO. Carbon monoxide conversion to acetate and hydrogen by *Desulfotomaculum* species has been reported (Henstra et al., 2007; Parshina et al., 2005; Plugge et al., 2002), and it is likely that the *Desulfotomaculum* sp. in T-Syn and T-Syn-CO cultures is growing on CO. The other predominant bacterium identified in T-Syn and T-Syn-CO enrichments is closest related to *Caloribacterium cisternae*, which is a recently described new species and genus of the *Thermoanaerobacteraceae* family (Slobodkina et al., 2012). The low 16S rRNA gene sequence identity of these microorganisms with *Caloribacterium cisternae* strain SGL43T (94%) makes it difficult to speculate about their physiological capabilities, especially with respect to the utilization syngas and CO. *C. cisternae* is not known to convert CO.

In T-CO, CO was first converted to H_2 and CO_2, and later acetate was formed. These results can be directly linked to the microbial community. According to the physiological characteristics of known *Thermincola* species (that are able to oxidize CO into H_2 and CO_2 (Sokolova et al., 2005)), it is plausible to infer that *Thermincola*-related bacteria is responsible for hydrogenogenic CO conversion in T-CO cultures. In fact, the band present in T-CO DGGE profile corresponding to a *Thermincola*-like organism is always present and intense when H_2 is produced (Figure 3.4). After extended incubation of T-CO, it was observed that the H_2 produced has been used for acetate conversion. DGGE profile of the same culture at this sampling point, showed an increase on the band intensity corresponding to the *Thermoanaerobacter*-like bacterium. Results from DGGE profiles together with physiological response of T-CO after extended incubation, strongly suggest that *Thermoanaerobacter*-related bacterium converts H_2/CO_2 to acetate. Although *T. thermohydrosulfuricus* strain $E100-69^T$ is not able to convert H_2/CO_2 to acetate (data not shown), *T. kivui* strain $LKT-1^T$ (also a CO-oxidizing bacterium) forms acetate from H_2/CO_2 (Kevbrina et al., 1996).

Bacteria of the genera *Desulfotomaculum* and *Thermincola*, and *Caloribacterium* and *Thermoanaerobacter* are associated with syngas and/or CO conversion in thermophilic enrichment cultures. Using the same thermophilic sludge, specialization of the microbial communities depended on the start-up of the experiments. Considering the possibility of using syngas for the production of biofuels, and specifically of a microbial process for obtaining H_2-enriched syngas, it could be interesting to previously submit anaerobic biomass to high CO partial pressures in order to select for hydrogenogenic microorganisms. Methane production should also be possible,

but more studies on CO toxicity are needed; longer incubation times might be necessary to avoid that methanogens are outcompeted by acetogenic bacteria. Syngas is produced at high temperatures (by gasification processes) and CO occurs naturally at high temperature environments, such as hot springs or volcanoes - more information about the microbiology of syngas and/or CO-conversion at thermophilic conditions can be retrieved from these environments. Moreover, studies on the behavior of anaerobic CO-converting mixed cultures in continuous operation are necessary.

Chapter 4

Description of *Thermoanaerobacter carboxyditolerans* sp. nov. and comparative analysis of carbon monoxide tolerance of *Thermoanaerobacter* species

A novel anaerobic, thermophilic, carbon monoxide-tolerant bacterium, strain PCO, was isolated from sludge of an anaerobic digester treating municipal solid waste. Vegetative cells were straight rods 0.2 to 0.5 µm in diameter and 1.5 to 3.5 µm in length, while sporulating cells were much longer (5 to 10 µm). The optimal temperature for growth was 70 °C. No growth was detected below 37 °C and above 75 °C. The optimal pH was between 6.5 and 7.5. The G+C content of the genomic DNA was 34.5 mol%. Based on 16S rRNA gene sequence analysis, this bacterium is most closely related to *Thermoanaerobacter thermohydrosulfuricus* (97% sequence identity). Strain PCO grows with a variety of mono-, di- and polysaccharides forming mainly lactate, ethanol, acetate and hydrogen. Anthraquinone-2,6-disulfonate (AQDS) and elemental sulfur could act as electron acceptors, but not nitrate and thiosulfate. Strain PCO is tolerant to a high partial pressure of CO (pCO = 1.7 bar, 100% CO). We also observed tolerance to CO for *Thermoanaerobacter thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* and *T. wiegelii*. Although growth of these bacteria was not substantially affected by CO, product formation was influenced by CO; hydrogen production decreased with increasing CO partial pressure. Based on 16S rRNA gene sequence analysis, physiological and biochemical characteristics, it is proposed that strain PCO^{T} should be classified in the genus *Thermoanaerobacter* as a new species, *Thermoanaerobacter carboxyditolerans* (=DSM 24750^{T} = CGMCC 1 5182^{T}).
The 16S rRNA gene sequence of *Thermoanaerobacter carboxyditolerans* strain PCO is available in the DDBL/EMBL/GenBank databases under the accession number HF586422.

4.1 Introduction

Thermophiles thrive at extreme conditions and their evolutionary significance and biotechnological potential have triggered microbiological and biotechnological research over the last decades (Turner et al., 2007; Wagner and Wiegel, 2008). Thermophilic microorganisms of the class Clostridia, such as members of the genera *Clostridium*, *Thermoanaerobacter*, *Thermoanaerobacterium* and *Caldicellulosiruptor*, can be used as catalysts for the production of biofuels or other chemicals of interest (Carere et al., 2012; Hemme et al., 2010). For instance, members of the *Thermoanaerobacter* genus can be utilized to produce ethanol and hydrogen from a variety of saccharides (Balk et al., 2009; Kim et al., 2001; Lee et al., 1993, 2007; Shaw et al., 2010; Wiegel and Ljungdahl, 1981). Some of members of this genus can also utilize less common substrates, such as carbon monoxide (CO). *T. thermohydrosulfuricus* subsp. *carboxydovorans* can grow with CO as sole electron donor, although no growth was observed for CO partial pressure higher than 0.25 bar (25% CO, total pressure 1 bar) (Balk et al., 2009). It converts CO mainly to H_2 and CO_2. Growth of *T. kiviu* with CO and acetate formation was also evidenced; CO was diluted with CO_2/H_2, using CO partial pressure in the range of 0.04 - 0.35 bar (9% CO_2; 38% H_2; 4, 11, 21 and 35% CO, and different N_2 percentages; total pressure was 1 bar) (Kevbrina et al., 1996). Additionally, growth was stimulated by the presence of H_2, compared to cultures supplemented with CO/CO_2 only, and acetate was the main product formed. The utilization of CO seems to be a strain specific trait. *T. thermohydrosulfuricus* shares 99% similarity of the 16S rRNA gene sequence and over 70% DNA-DNA hybridization with *T. thermohydrosulfuricus* subsp. *carboxydovorans*, but only the last one can use CO. The other known *Thermoanaerobacter* species are not known to grow or use CO, neither it is known if these species can endure the presence of CO. As *Thermoanaerobacter* species are able to form hydrogen as product, CO can have an inhibitory effect. CO is a well known inhibitor of hydrogenases (Bennett et al., 2000).

In this work, isolation and characterization of a new CO-tolerant *Thermoanaerobacter* sp., strain PCO^T, is reported. This strain was isolated from a syngas degrading culture that was enriched from biomass of a solid waste anaerobic digester operated at 55 °C. The effect of CO on growth and product formation of strain PCO and other *Thermoanaerobacter* species was also studied.

4.2 Materials and Methods

4.2.1 Enrichments and isolation

Microbial cultures were enriched with syngas (mixture of CO, CO_2 and H_2) as sole carbon and energy source. Cultures were repeatedly transferred. Total pressure in the bottles' headspace was 1.7 bar and CO percentage varied from 5 to 50% (pCO/P). Bottles were incubated in the dark at 55 °C while shaken at 120 rpm. An anaerobic, phosphate-buffered mineral salt medium

was used for enrichment and isolation of strain PCO with pyruvate (20 mM) as substrate. The phosphate-buffered mineral medium contained the following components (per liter): Na_2HPO_4, 1.63 g; NaH_2PO_4, 1.02 g; resazurin, 0.5 g; NH_4Cl, 0.3 g; $CaCl_2 \cdot 2\,H_2O$, 0.11 g; $MgCl_2 \cdot 6\,H_2O$, 0.10 g; NaCl, 0.3 g; 1 mL of acid and alkaline trace element stock each, and 0.2 ml of vitamin stock. Medium was reduced with 0.8 mM sodium sulfide (final concentration) before inoculation. Trace elements and vitamins were prepared as described previously (Stams et al., 1993). Isolation of strain PCO was done using soft agar (1.5%, w/v) inoculations and by serial dilutions in liquid medium. Sodium pyruvate was added from a 1 M filter-sterilized stock solution. Purity of the culture was checked by microscopic examination after growth with different substrates (Olympus CX41, Tokyo, Japan). Direct sequencing of the 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) were also applied to check the genetic purity of the culture.

4.2.2 DNA isolation, PCR and DGGE

Total genomic DNA from cultures of strain PCO was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA), according to the manufacturer's instructions. 16S rRNA gene was directly amplified from genomic DNA by PCR, using the primer set 027F/1492R (Nübel et al., 1996) and the following PCR program: pre-denaturation, 2 min at 95 °C; 30 cycles of denaturation, 30 s at 95 °C, annealing, 40 s at 52 °C and elongation, 90 s at 72 °C; and post-elongation, 5 min, at 72 °C. For DGGE analysis 16S rRNA gene was partially amplified from genomic DNA with primer set U968GC-f/L1401-r (Lane, 1991; Muyzer et al., 1993). The thermo cycling program used for PCR-DGGE amplification was: pre-denaturation, 5 min at 95 °C; 35 cycles of denaturation, 30 s at 95 °C, annealing, 40 s at 56 °C and elongation, 90 s at 72 °C; and post-elongation, 5 min at 72 °C. DGGE was performed using a DCode system (Bio-Rad, Hercules, CA, USA). Gels contained 8% (wt/vol) polyacrylamide (37.5 : 1 acrylamide/bis-acrylamide) and a linear denaturing gradient of 30−60%, with 100% of denaturant corresponding to 7 M urea and 40% (vol/vol) formamide. Electrophoresis was performed for 16 h at 85 V and 60 °C in a 0.5× Tris-Acetate-EDTA buffer. DGGE gels were stained with silver nitrate (Sanguinetti et al., 1994).

4.2.3 Sequencing and phylogenetic analysis

PCR products obtained from 16S rRNA gene amplification were purified using the PCR Clean Up kit NucleoSpin Extract II (Macherey-Nagel, Düren, Germany) and sequenced directly at Eurofins MWG Operon (Ebersberg, Germany). Partial sequences were assembled using the alignment editor BioEdit v7.0.9 software package (Hall, 1999). Similarity searches for the 16S rRNA gene sequence derived from strain PCO were performed using the NCBI BLAST search program within the GenBank database (Altschul et al., 1990). Alignment of the 16S rRNA sequences was performed by using the FastAligner V1.03 tool of the ARB program package (Ludwig et al., 2004). The neighbor joining method (Saitou and Nei, 1987) was used for the

construction of a 16SrRNA gene based phylogenetic tree.

4.2.4 Physiological characterization

Cell morphology of strain PCO was examined by phase contrast microscopy (Leica DM 2000, Wetzlar, Germany). The optimal temperature and optimal pH for growth of strain PCO were determined by measuring optical absorbance at 600 nm with a spectrophotometer (U-1500 Hitachi, Tokyo, Japan). Unless otherwise stated, all physiological tests of strain PCO and the close relatives (*T. thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* and *T. wiegelii*) were performed using a bicarbonate-buffered mineral salt medium (Stams et al., 1993) incubated at 55 °C for up to 15 days. The type strains of *Thermoanaerobacter thermohydrosulfuricus* (DSM 527^{T}), *T. brockii* subsp. *finnii* (DSM 3389^{T}), *T. pseudethanolicus* (DSM 2355^{T}) and *T. wiegelii* (DSM 10319^{T}) were obtained from DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany).

Growth of the strains on different carbon sources and electron acceptors was examined visually and under the microscope. Pyruvate (20 mM) was used as carbon source for testing the utilization of different electron acceptors and the optimum growth temperature (from 20 to 85 °C) and pH (from 5.7 to 8.0) for strain PCO. For testing the utilization of different electron acceptors by *T. thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* and *T. wiegelii*, glucose (20 mM) was used as carbon source. Sensitivity to antibiotics by strain PCO was tested using glucose as carbon source. Antibiotics were added from freshly prepared anoxic filter-sterilized solutions to a final concentration of 100 μg mL^{-1}. Cells from active cultures of strain PCO were stained using Gram staining techniques. G+C content determination and cellular fatty acids composition were performed by the identification service of the DSMZ (Braunschweig, Germany).

4.2.5 Analytical methods

Soluble substrates and intermediates (sugars, volatile fatty acids and alcohols) were measured using a HPLC Thermo Electron equipment with a Shodex SH1821 column and equipped with a RI detector. The mobile phase used was sulfuric acid (0.01 N) at a flow rate of 0.6 mL min^{-1}. Column temperature was set at 60 °C. Ionic species were analyzed by chromatography using a HPLC Dionex system, equipped with an Ionpac AS22 column and ED40 electrochemical detector. Column temperature and pressure varied between 35-40 °C and 130-160 bar. Gaseous compounds (CO, CO_2, H_2) were analyzed by gas chromatography on a GC-2014 Shimadzu with a thermal conductivity detector. CO_2 was analyzed with a CP Poraplot Q column (25 m length, 0.53 mm internal diameter; film thickness, 20 μm). Helium was used as carrier gas at a flow rate of 15 mL min^{-1}, and the temperatures in the injector, column and detector were 60, 33 and 130 °C. CO and H_2 were analyzed with a Molsieve 13X column (2 m length, 3 mm internal diameter). Argon was used as carrier gas at a flow rate of 50 mL min^{-1}, and temperatures in

Table 4.1: Stoichiometry of glucose conversion to hydrogen, lactate, ethanol, acetate and 1,3 PDO.

Complete conversion	$C_6H_{12}O_6 + 6\,H_2O \longrightarrow 6\,CO_2 + 12\,H_2O$
Lactate formation	$C_6H_{12}O_6 \longrightarrow 2\,C_3H_6O_3$
Ethanol formation	$C_6H_{12}O_6 \longrightarrow 2\,C_2H_6O + 2\,CO_2$
Acetate formation	$C_6H_{12}O_6 + 2\,H_2O \longrightarrow 2\,C_2H_4O_2 + 2\,CO_2 + 4\,H_2$
1,3 PDO formation	$C_6H_{12}O_6 + 4\,H_2 \longrightarrow 2\,C_3H_8O_2 + 2\,H_2O$

the injector, column and detector were 80, 100 and 130 °C.

4.2.6 Carbon monoxide tolerance tests

Strain PCO, *Thermoanaerobacter thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* and *T. wiegelii* were tested for CO tolerance. All the cultures were incubated with 0%, 50% and 100% CO (pCO/P; P = total pressure) in the headspace. Additionally, strain PCO was incubated with 25% and 75% CO. Initial total pressure was 1.7 bar in all the assays; N_2 was used to pressurize the bottles' headspace for CO percentages lower than 100%. The tests were performed using an anaerobic phosphate-buffered mineral salt medium and glucose (20 mM) was used as carbon and energy source. Bottles were incubated in the dark, at 55 °C and shaken at 120 rpm. Growth of different strains was determined by measuring optical density at 600 nm with a spectrophotometer (U-1500 Hitachi, Tokyo, Japan). The statistical significance of the differences detected in both glucose degradation rates and end products production was evaluated using single factor analysis of variances (ANOVA).

4.2.7 Stoichiometry, products yields, carbon recovery and oxidation-reduction balance calculations

The stoichiometry of glucose conversion to hydrogen, lactate, ethanol, acetate and 1,3-propanediol (1,3 PDO) are presented in Table 4.1. The yields were calculated from the plateau of the cumulative production of each product (mM) subtracted by the value measured at t=0, divided by the difference between the initial and final glucose concentration.

The carbon recovery (CR) was calculated by the sum of the carbon moles in all the products divided by the carbon moles in the glucose consumed (eq. 4.1).

$$CR(\%) = \frac{\sum_i^N Ci\left[(P_i)_f - (P_i)_0)\right]}{6\left[(glucose)_0 - (glucose)_f\right]} \times 100 \tag{4.1}$$

Where,
i - refers to a specific product and the sum is extended to all the products containing carbon
C_i - is the number of carbon moles in the product i

f - refers to final values (average of two or three last values)
0 - refers to initial values
N - is the number of products considered (4)

The oxidation-reduction balance was calculated taking into account the measured H_2 and the H_2 producing and consuming reactions (acetate and 1,3 PDO formation reactions, respectively). According to these reactions, and taking into consideration the products distribution, a given H_2 concentration would be expected. The difference between the produced H_2 effectively measured and the H_2 that would result from the production of acetate and 1,3-PDO divided by the concentration of glucose converted, represents the shift to the 100% balance. Considering the theoretical moles of H_2 that would result from the stoichiometric conversion of glucose to H_2 and CO_2 (12), the % of H_2 recovery, or oxidation-reduction balance, can be calculated from eq. 4.2:

$$\frac{12 + \frac{\Big[(H_2)_f - (H_2)_0\Big] - \Big[4 \times \Big[(acetate)_f - (acetate)_0\Big] - 4 \times \Big[(1,3-PDO)_f - (1,3-PDO)_0\Big]\Big]}{\Big[(glucose)_0 - (glucose)_f\Big]}}{12} \times 100 \tag{4.2}$$

4.3 Results and Discussion

4.3.1 Isolation and characterization of strain PCO^T

An anaerobic pyruvate-fermenting bacterium, strain PCO, was isolated from a culture enriched with syngas as sole carbon source and energy substrate. Strain PCO has a G+C content of the DNA of 34.5 mol % and shares 97% identity with the 16S rRNA gene of *Thermoanaerobacter thermohydrosulfuricus* (Fig. 4.1).

Strain PCO stained Gram negative in both the early exponential and the late stationary phase. Cells of strain PCO are straight rods with variable size (approx. 0.2 to 0.5 µm in diameter and 1.5 to 3.5 µm in length) and usually cells occur singly. Strain PCO formed terminal round endospores, which is a characteristic of *Thermoanaerobacter* species (Balk et al., 2009; Kim et al., 2001; Lee et al., 1993, 2007; Shaw et al., 2010; Wiegel and Ljungdahl, 1981). Sporulating cultures contained somewhat thinner and more elongated cells (approx. 0.2 to 0.4 µm in diameter and 5 to 10 µm in length (Fig. 4.2).

When grown in soft-agar, strain PCO formed white and round colonies inside the agar. Strain PCO had an optimum growth temperature of 70 °C. No growth was detected below 37 °C and above 75 °C. The optimum pH of growth of strain PCO was between 6.5 and 7.5. Streptomycin at 100 µg mL^{-1} did not inhibit growth, but penicillin, ampicillin, chloramphenicol and kanamycin completely inhibited growth at a concentration of 100 µg mL^{-1}. The fatty acid composition of strain PCO was dominated by iso-C16:0 (35.57%) and C16:0 (19.84%) (Table 4.2).

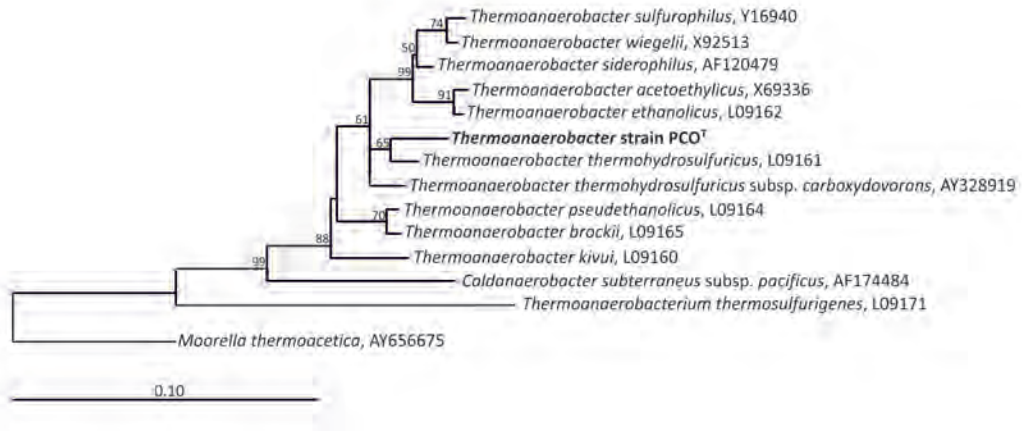


Figure 4.1: Phylogenetic tree of 16S rRNA gene sequences showing the position of strain PCO^T relative to other species of the genus *Thermoanaerobacter* as well as selected reference sequences of related bacteria. The phylogentic tree was calculated using the ARB software package (Saitou and Nei, 1987) and applying the neighbor-joining method with Felsenstein correction. The significance of each branch is indicated at the nodes by bootstrap values (%) based on 1000 replications; only values above 50% are given. GenBank accession numbers of 16S rRNA gene sequences are indicated. Bar, 10% sequence divergence.

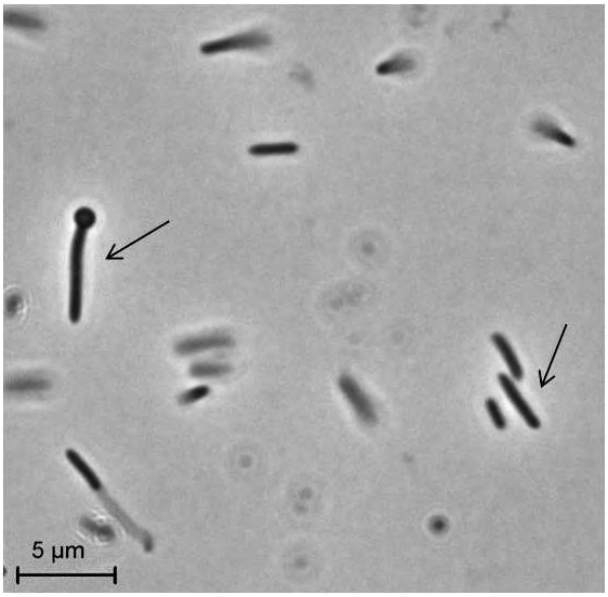


Figure 4.2: Phase-contrast micrograph of strain PCO^T, showing vegetative and sporulating cells. Size bar indicates 5 µm.

Table 4.2: Cellular fatty acid composition (%) of strain PCO^T.

Fatty acids	**Strain** PCO^T
C12:0	0.64
C13:1	0.42
iso-C14:0	3.92
C14:0	4.78
iso-C13:0 3OH	0.77
iso-C15:0	7.20
C15:0	4.13
iso-C14:0 3OH	1.35
C14:0 2OH	1.11
iso-C16:0	35.57
C16:0	19.84
iso-C17:0	3.27
C17:0	3.33
iso-C18:0	0.72
C18:0	2.71
C20:4	1.35

In contrast, the most abundant fatty acids of *Thermoanaerobacter thermohydrosulfuricus*, the phylogenetically closest relative of strain PCO, were iso-C15:0 and iso-C17:0 (Balk et al., 2009).

Strain PCO could utilize the following substrates (at a concentration of 20 mM unless indicated otherwise): arabinose, cellobiose, cellulose ($5\,g\,L^{-1}$), fructose, galactose, glucose, lactose, maltose, mannitol, mannose, pectin ($5\,g\,L^{-1}$), pyruvate, raffinose, ribose, sorbitol, starch ($5\,g\,L^{-1}$), sucrose, trehalose, xylan ($5\,g\,L^{-1}$), xylose and yeast extract ($5\,g\,L^{-1}$). The doubling time of strain PCO with glucose was 1.03 ± 0.03 days. No growth occurred with acetate, ethanol, formate, glycerol, glycine, lactate, methanol, propionate, CO (from 20 to 100% CO, 1.7 bar) and H_2/CO_2 (80/20%, 1.7 bar). Yeast extract or other co-factors were not needed for growth. The main products detected from glucose fermentation by strain PCO were lactate, ethanol, acetate and H_2, which are typically formed by most of the *Thermoanaerobacter* species (Balk et al., 2009; Kim et al., 2001; Lee et al., 1993, 2007; Shaw et al., 2010; Wiegel and Ljungdahl, 1981). Strain PCO converted 1 mol of glucose to 0.92 ± 0.11 mol of lactate, 0.39 ± 0.07 mol of ethanol, 0.29 ± 0.04 mol of acetate, 0.22 ± 0.04 mol of H_2 and 0.21 ± 0.05 mol of 1,3-propanediol (Table 4.4 and Figure 4.3).

Strain PCO is not able to convert CO, but as will be described below, strain PCO changes its metabolism in the presence of CO, although the growth rate and glucose conversion rates are not affected. Strain PCO is able to reduce elemental sulfur (2% (w/v)) and AQDS (20 mM). Sulfate (20 mM), sulfite (10 mM), thiosulfate (20 mM), nitrate (20 mM) and nitrite (10 mM) could not serve as electron acceptors. The comparison between the morphological, biochemical

Table 4.3: Physiological and biochemical characteristics of (1) strain PCO^T and phylogenetic related species, (2) *Thermoanaerobacter thermohydrosulfuricus* (DSM 527^T), (3) *T. brockii* subsp. *finnii* (DSM 3389^T), (4) *T. pseudethanolicus* (DSM 2355^T) and (5) *T. wiegelii* (DSM 10319^T). Data are from Cayol et al. (1995); Lee et al. (1993); Onyenwoke et al. (2007); Cook et al. (1996) and this study. Abbreviation: nr, not reported. Symbols: +, utilized; +/−, poorly utilized; −, not utilized; ∗, data from this study.

Characteristics	**1***	**2**	**3**	**4**	**5**
Growth pH (optimum)	6.5 – 7.5	6.9 – 7.5	6.5 – 6.8	nr	6.8
Growth temperature (optimum, °C)	70	67 – 69	65	65	65 – 68
Spore formation	+	+	+	+	+
Gram reaction	negative	variable	variable	variable	negative
DNA G+C content (mol%)	34.5	37.6	32	34.4	35.6
Substrate utilization:					
acetate	-	-	-*	-*	-*
arabinose	+	+/-	-*	-	-
carboxymethylcellulose	+/-	+	-*	-*	+*
cellobiose	+	+	+	+	+
cellulose	+	-	+/-*	-*	+*
CO	-	-	-*	-*	-*
ethanol	-	-*	-*	-*	-
formate	-	-*	-*	-*	-*
fructose	+	+	+	-*	+
galactose	+	+	+	+*	+
glucose	+	+	+	+	+
glycerol	-	-	+/-*	-	+
glycine	-	+/-*	-*	-*	+/-*
H_2/CO_2	-	-*	-*	-	-*
lactate	-	-	-*	-*	-
lactose	+	+*	+	-*	+
maltose	+	+	+	+	+
mannitol	+	+/-	+	-	+
mannose	+	+	+	+/-*	+
methanol	-	-*	-*	-*	-*
pectin	+	+	+/-*	-*	+
peptone	+/-	+	-*	+/-*	+*
propionate	-	-*	-*	-*	-*
pyruvate	+	+	+	+	-
raffinose	+	+*	+*	+*	+
ribose	+	+	+	+	-
sorbitol	+	+*	+/-*	+/-*	+*
starch	+	+	+*	+	+
succinate	-	-*	-*	-*	-
sucrose	+	+	+	+	+
trehalose	+	+	+*	+*	+*
xylan	+	+	+*	+*	+*
xylose	+	+	+	+	+
yeast extract	+	+	-*	-*	+*
Electron acceptors:					
AQDS	+	-*	+*	+*	+*
elemental sulfur	+	+*	+*	+*	+*
nitrate	-	+	-*	-*	-*
nitrite	-	-	-*	-*	-*
sulfate	-	-	-*	-*	-*
sulfite	-	+	-*	-*	+*
thiosulfate	-	+	+	+	+*

and physiological characteristics of strain PCO^T and its closest relatives is presented in Table 4.3. All of them can use thiosulfate as electron acceptor, but strain PCO cannot. In addition, strain PCO can be differentiated from *T. thermohydrosulfuricus*, because of the differences in the lipid composition, and its ability to grow and ferment cellulose and reduce AQDS. In contrast, *T. thermohydrosulfuricus* can also use nitrate and sulfite as electron acceptor, but strain PCO cannot. Based on phylogenetic and physiological results, we propose that strain PCO^T represents a novel species of *Thermoanaerobacter* genus, for which the proposed name is *Thermoanaerobacter carboxyditolerans* sp. nov.

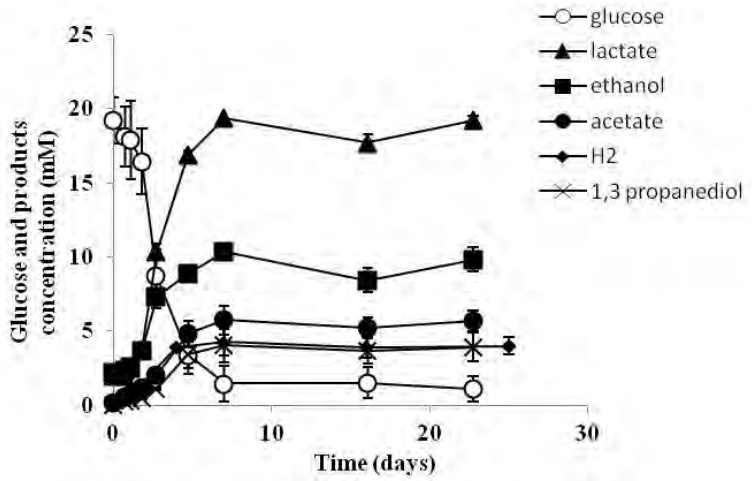


Figure 4.3: Glucose conversion by strain PCO^T over time. The results represent the average of duplicate experiments.

4.3.2 Comparative analysis of CO tolerance of strain PCO^T and other *Thermoanaerobacter* species

Although strain PCO^T was isolated from a syngas degrading culture, it did not utilize any syngas component for growth. However, when strain PCO^T was cultured with 20 mM of glucose and subjected to different CO concentrations in the headspace (0, 25, 50, 75 and 100%) no significant differences were found in glucose consumption rates (data not shown). Concentrations of CO of 75% and lower, did not affect substantially product formation from glucose, except for hydrogen. Hydrogen production by strain PCO^T decreased significantly ($P = 0.0001$) from 3.94 ± 0.55 mM to 1.52 ± 0.13 mM when cultures were incubated with 0 and 25% CO in the headspace, respectively (Fig. 4.4A). Gradual increments on CO partial pressure up to 100% caused a decrease in H_2 production, with H_2 production of < 0.02 mmol per liter at 100% CO. The other end products from glucose conversion were only affected when the culture was growing with 100% CO in the headspace. There was a significantly decrease on the final production of lactate ($P = 0.01$) and ethanol ($P = 0.004$) only when comparing cultures grown with 75 and

100% CO (Figs 4.4A and 4.5A). Strain PCO^T changed its metabolism as a response to the presence of CO, but growth was not inhibited.

T. thermohydrosulfuricus subsp. *carboxydovorans* and *T. kiviu* are able to use CO, but for most *Thermonaerobacter* species the ability to use CO was never tested. The ability to use CO and the tolerance to CO by *T. thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* and *T. wiegelii* was tested. None of the tested *Thermoanaerobacter* species can utilize CO, resembling strain PCO^T (Table 4.3). All *Thermoanaerobacter* strains tested could grow and completely convert glucose in the presence of 0, 50 or 100% of CO in the headspace (Table 4.4, Figs. 4.4 and 4.5). In each case, the carbon recovery at the end of the incubations was nearly 100% (Table 4.4). All the tested strains produced the same end products from glucose fermentation, i.e. hydrogen, lactate, acetate, ethanol and 1,3-propanediol. Nevertheless, final product concentrations (Figs. 4.4 and 4.5) varied for the different tested strains. Ethanol production was in general less affected by the presence of CO at different concentrations. A significant decrease in ethanol production was only observed in the presence of 100% CO and just for strain PCO^T ($P = 0.004$) and for *T. wiegelii* ($P = 0.002$). Conversely, and as already reported for strain PCO^T, H_2 production by the tested strains decreased significantly, even in the presence of low CO concentrations. In the presence of 100% CO, H_2 production by the different *Thermoanaerobacter* species decreased by 75-95%. Acetate was also formed to a lesser concentration with increasing CO percentage in cultures' headspace (reduction between 25-50%); the exception was strain PCO^T for which acetate final concentration increased significantly in the presence of high CO percentage (acetate final concentration in cultures with 0% and 100% CO were 5.4 ± 0.6 mM and 8.3 ± 0.5 mM, respectively ($P = 0.0003$)). Final concentration of 1,3-propanediol had 1-2 time fold increase in the presence of 100% CO for the different tested strains. For instance, 1,3-propanediol production by strain PCO^T increased from 3.8 ± 0.8 mM (in the absence of CO) to 7.2 ± 0.6 mM (with 100% CO in the headspace).

The results point to metabolic changes in versatile thermophilic microorganisms such as *Thermoanaerobacter* species upon addition of CO, resulting in a general decrease in H_2 production and in an augmented generation of 1,3 propanediol. Although tested *Thermoanaerobacter* strains – strain PCO^T, *T. thermohydrosulfuricus*, *T. brockii* subsp. *finnii*, *T. pseudethanolicus* and *T. wiegelii* – could not utilize CO, all were able to withstand the presence of this compound by diverting their metabolic routes from H_2 production. This most likely indicates that CO is toxic to the hydrogenases present in *Thermoanaerobacter* species but, nevertheless, these microorganisms can tolerate CO to high concentrations.

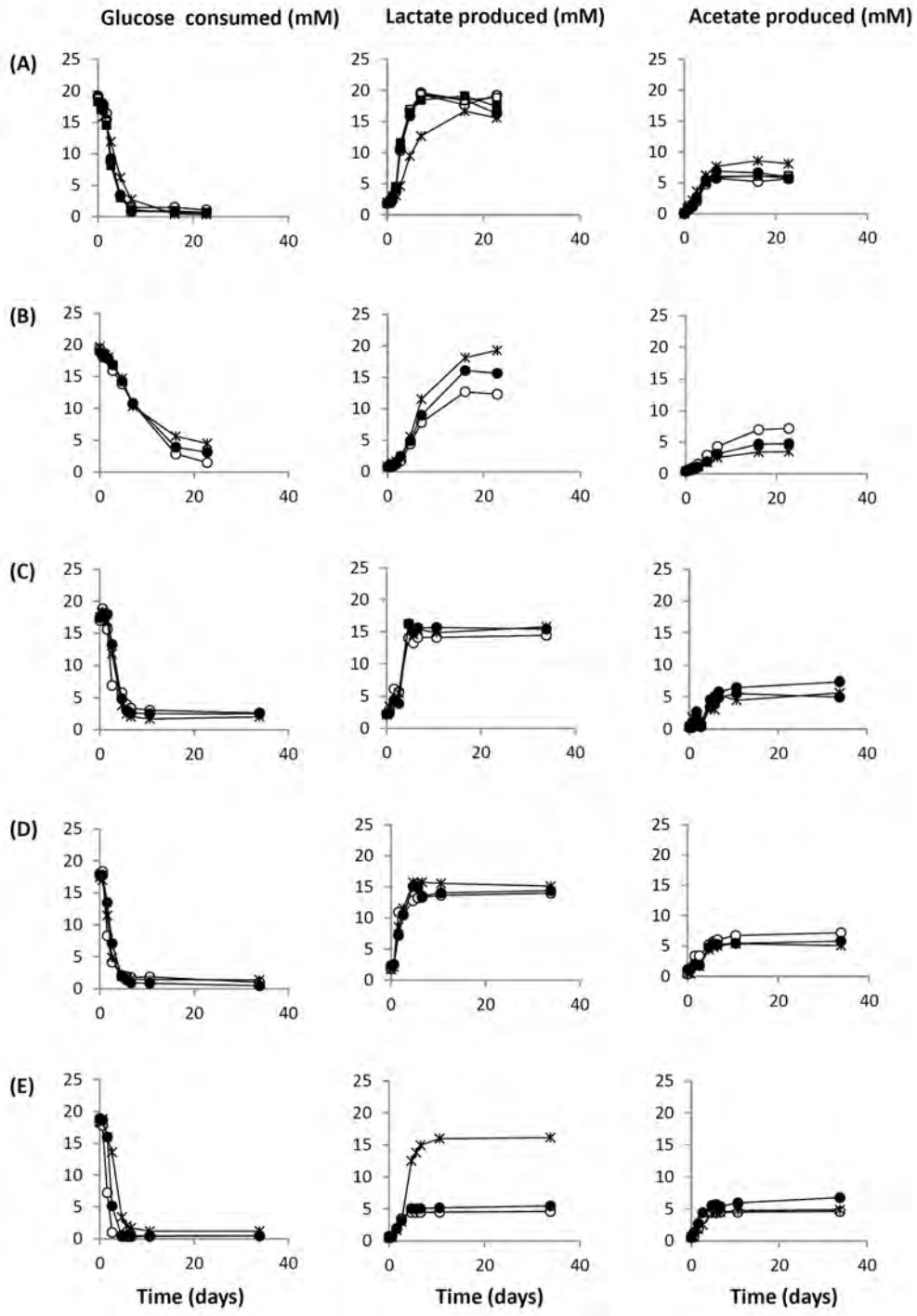


Figure 4.4: Glucose conversion and VFA's production (lactate and acetate) over time by (A) strain PCO^{T}, (B) *Thermoanaerobacter thermohydrosulfuricus* (DSM 527^{T}), (C) *T. brockii* subsp. *finnii* (DSM 3389^{T}), (D) *T. pseudethanolicus* (DSM 2355^{T}) and (E) *T. wiegelii* (DSM 10319^{T}). Each graph shows the results obtained for the conditions tested (0, 25, 50, 75 or 100% CO in gas phase). Plotted are the average data of duplicate experiments. Symbols: (○) 0% CO, (square) 25% CO (pCO = 0.43 bar), (●) 50% CO (pCO = 0.85 bar), (black square) 75% CO (pCO = 1.28 bar) and (*) 100% CO (pCO = 1.7 bar).

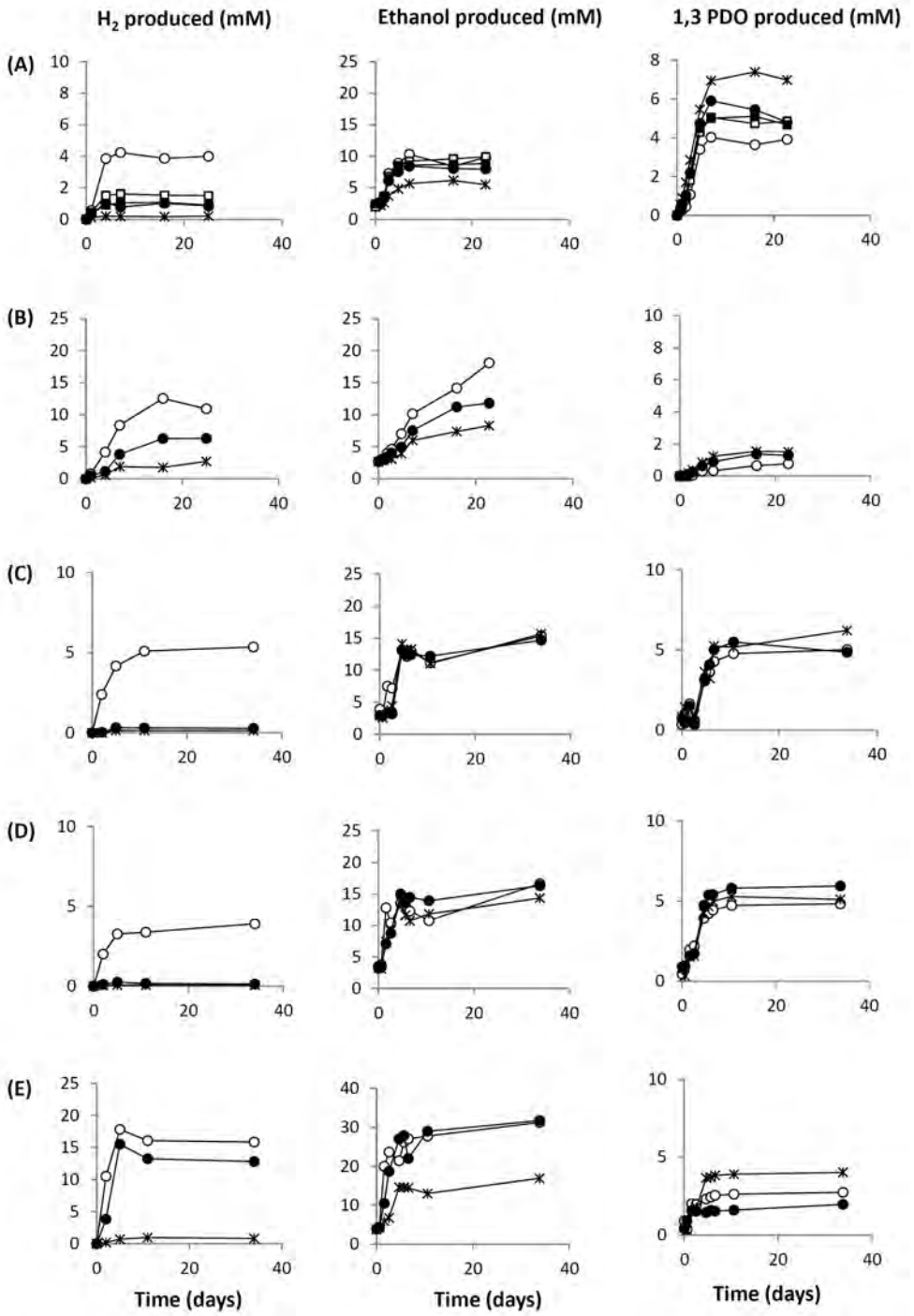


Figure 4.5: H_2 and alcohols (ethanol and 1,3-propanediol) production from glucose conversion over time by (A) strain PCO^T, (B) *Thermoanaerobacter thermohydrosulfuricus* (DSM 527^T), (C) *T. brockii* subsp. *finnii* (DSM 3389^T), (D) *T. pseudethanolicus* (DSM 2355^T) and (E) *T. wiegelii* (DSM 10319^T). Each graph shows the results obtained for the conditions tested (0, 25, 50, 75 or 100% CO in gas phase). Plotted are the average data of duplicate experiments. Symbols: (○) 0% CO, (square) 25% CO (pCO = 0.43 bar), (●) 50% CO (pCO = 0.85 bar), (blacksquare) 75% CO (pCO = 1.28 bar) and (*) 100% CO (pCO = 1.7 bar).

Table 4.4: Effect of CO partial pressure on glucose conversion by strain PCO^{T}, *Thermoanaerobacter thermohydrosulfuricus* (DSM 527^{T}), *T. brockii* subsp. *finnii* (DSM 3389^{T}), *T. pseudethanolicus* (DSM 2355^{T}) and *T. wiegelii* (DSM 10319^{T}).

Strains	**CO (%)**	**glucose consumed (mM)**	**mol H_2/mol glucose**	**mol acetate/mol glucose**	**mol lactate/mol glucose**	**mol ethanol/mol glucose**	**mol 1,3-propanediol/mol glucose**	**Carbon recovery (%)**	**Oxidation-reduction balance (%)**
Strain PCO^{T}	0	17.9 ± 1.80	0.22 ± 0.04	0.29 ± 0.04	0.92 ± 0.11	0.39 ± 0.07	0.21 ± 0.05	90.6 ± 9.8	100.5 ± 0.9
	25	18.0 ± 0.80	0.08 ± 0.01	0.33 ± 0.03	0.93 ± 0.05	0.43 ± 0.02	0.27 ± 0.03	97.7 ± 4.8	99.6 ± 0.6
	50	18.3 ± 1.24	0.05 ± 0.01	0.35 ± 0.06	0.85 ± 0.14	0.33 ± 0.04	0.28 ± 0.05	90.2 ± 9.5	99.3 ± 1.2
	75	17.7 ± 1.54	0.06 ± 0.01	0.34 ± 0.06	0.93 ± 0.12	0.36 ± 0.07	0.28 ± 0.06	95.1 ± 10.1	99.5 ± 1.2
	100	18.2 ± 0.22	< 0.02	0.46 ± 0.03	0.78 ± 0.05	0.21 ± 0.02	0.40 ± 0.03	92.7 ± 3.4	99.0 ± 0.7
T. thermohydrosulfuricus	0	17.8 ± 1.00	0.61 ± 0.06	0.38 ± 0.06	0.64 ± 0.09	0.86 ± 0.14	0.04 ± 0.01	96.4 ± 8.8	99.5 ± 1.1
	50	15.7 ± 0.90	0.40 ± 0.08	0.28 ± 0.04	0.94 ± 0.14	0.58 ± 0.08	0.08 ± 0.01	94.1 ± 9.1	100.1 ± 0.9
	100	15.2 ± 0.30	0.18 ± 0.01	0.20 ± 0.01	1.21 ± 0.05	0.37 ± 0.06	0.10 ± 0.01	94.4 ± 3.8	99.8 ± 0.2
T. brockii subsp. finnii	0	14.2 ± 1.80	0.37 ± 0.05	0.48 ± 0.07	0.85 ± 0.11	0.66 ± 0.19	0.32 ± 0.04	115.1 ± 16.0	100.5 ± 0.8
	50	14.9 ± 0.90	0.02 ± 0.002	0.32 ± 0.04	0.89 ± 0.06	0.70 ± 0.13	0.30 ± 0.04	111 ± 8.3	99.7 ± 0.8
	100	15.7 ± 0.40	< 0.02	0.30 ± 0.05	0.84 ± 0.04	0.66 ± 0.18	0.31 ± 0.06	105.8 ± 8.2	100.2 ± 1.2
T. pseudethanolicus	0	16.6 ± 0.50	0.22 ± 0.02	0.40 ± 0.02	0.72 ± 0.03	0.63 ± 0.22	0.26 ± 0.01	100.6 ± 8.7	99.6 ± 0.4
	50	17.1 ± 0.60	< 0.02	0.26 ± 0.07	0.70 ± 0.06	0.68 ± 0.10	0.29 ± 0.04	96.4 ± 6.5	100.5 ± 1.4
	100	16.0 ± 1.60	< 0.02	0.29 ± 0.04	0.85 ± 0.09	0.61 ± 0.12	0.28 ± 0.04	101.8 ± 11.2	99.8 ± 0.6
T. wiegelii	0	17.7 ± 1.80	0.90 ± 0.17	0.39 ± 0.11	0.22 ± 0.04	1.45 ± 0.22	0.10 ± 0.05	107.7 ± 13.0	102.6 ± 1.8
	50	18.5 ± 1.20	0.71 ± 0.12	0.32 ± 0.03	0.26 ± 0.02	1.43 ± 0.13	0.07 ± 0.02	104.2 ± 7.6	101.8 ± 1.1
	100	17.1 ± 1.70	0.05 ± 0.02	0.25 ± 0.08	0.91 ± 0.21	0.66 ± 0.26	0.21 ± 0.06	101.1 ± 8.3	94.3 ± 27.8

4.3.3 Description of *Thermoanaerobacter carboxyditolerans* sp. nov.

Thermoanaerobacter carboxyditolerans (car.bo.xy.di.tol.er'ans. N.L. neut. n. *carboxydum* carbon monoxide; L. part. adj. *tolerans* tolerating, enduring; N.L. part. adj. *carboxyditolerans* bacterium that tolerates carbon monoxide).

Cells are straight rods and show a variable shape: non-sporulating cells are short rods (approx. 0.2 to 0.5 µm in diameter and 1.5 to 3.5 µm in length), and sporulating cultures contain thinner and more elongated cells (approx. 0.2 to 0.5 µm in diameter and 5 to 10 µm in length). Usually cells occur singly. Cells produce terminal, round endospores and stain Gram negative. The most abundant fatty acids are iso-C16:0 (35.57%) and C16:0 (19.84%). The G+C content of the DNA of strain PCO is 34.5 mol %. The optimum temperature for growth is 70 °C. No growth was detected below 37 °C and above 75 °C. Optimum growth occurs at pH 6.5 to 7.5. Penicillin, ampicillin, chloramphenicol and kanamycin completely inhibited growth (100 µg mL^{-1}). Streptomycin at 100 µg mL^{-1} did not inhibit growth. Strain PCO^T grows with many substrates, tested in a concentration of 20 mM (unless indicated otherwise): arabinose, cellobiose, cellulose (5 g L^{-1}), fructose, galactose, glucose, lactose, maltose, mannitol, mannose, pectin (5 g L^{-1}), pyruvate, raffinose, ribose, sorbitol, starch (5 g L^{-1}), sucrose, trehalose, xylan (5 g L^{-1}), xylose and yeast extract (5 g L^{-1}). Glucose is fermented to mainly lactate and ethanol, but also to acetate, hydrogen and 1,3 propanediol. Strain PCO^T does not require yeast extract for growth. The following substrates do not support growth of strain PCO^T: acetate, ethanol, formate, glycerol, glycine, H_2/CO_2, CO, lactate, methanol, propionate and succinate. Strain PCO^T is tolerant to 100% of CO in the gas phase (pCO = 1.7 bar). Nitrate, nitrite, sulfate, sulfite and thiosulfate could not be used as electron acceptor, but AQDS and elemental sulfur were used as electron acceptors. The closest phylogenetic relative of strain PCO^T (based on 16S rRNA gene) is *Thermoanaerobacter thermohydrosulfuricus* (97% identity). The type strain is PCO^T (= DSM 24750^T = CGMCC $1\,5182^T$). It was isolated in Braga, Portugal from a syngas-degrading culture enriched from suspended sludge from an anaerobic municipal solid waste digester operated at 55 °C (Barcelona, Spain).

Chapter 5

Moorella stamsii sp. nov., a new anaerobic thermophilic hydrogenogenic carboxydotroph isolated from digester sludge

A novel anaerobic, thermophilic, carbon monoxide-utilizing bacterium, strain E3-O, was isolated from anaerobic sludge of a municipal solid waste digester. Cells were straight rods, 0.6 to 1 µm in diameter and 2 to 3 µm in length, growing as single cells or in pairs. Cells formed round terminal endospores. The temperature range for growth was 50 to 70 °C, with an optimum at 65 °C. The pH range for growth was 5.7 to 8.0, with an optimum at 7.5. Strain E3-O had the capability to ferment various sugars, such as fructose, galactose, glucose, mannose, raffinose, ribose, sucrose and xylose, producing mainly H_2 and acetate. In addition, the isolate was able to grow with CO as the sole carbon and energy source. CO oxidation was coupled to H_2 and CO_2 formation. The G + C content of the genomic DNA was 54.6 mol%. Based on 16S rRNA gene sequence analysis, this bacterium is most closely related to *Moorella glycerini* (97% sequence identity). Based on the physiological features and phylogenetic analysis, it is proposed that strain E3-O should be classified in the genus *Moorella* as a new species, *Moorella stamsii*. The type strain of *Moorella stamsii* is E3-O^T (=DMS 26271^T = CGMCC 1.5181^T). The Genbank/EMBL/DDBJ accession number for 16S rRNA gene sequence of *Moorella stamsii* strain E3-O is HF563589.

5.1 Introduction

Carbon monoxide is a gas present in natural and anthropogenic environments that is involved in several important redox reactions. CO metabolism is a significant part of the global carbon cycle. The number of known carboxydotrophic anaerobes is increasing in recent years due to their important role in CO conversion (Sokolova et al., 2009). CO is a potent electron donor (thermodynamic CO_2/CO redox potential = −520 mV) and represents an excellent source of energy for anaerobic microorganisms (Kochetkova et al., 2011; Oelgeschläger and Rother, 2008). Nevertheless, only few anaerobes have been described that exhibit the capacity for hydrogenogenic carboxydotrophy. Hydrogenogenic CO-oxidizing prokaryotes are able to grow on CO with the production of hydrogen and CO_2, according to the water-gas shift reaction: $CO + H_2O \longrightarrow H_2 + CO_2$ (Δ G = −20 kJ/mol) (Svetlitchnyi et al., 2001). The first organism described to perform this reaction in the dark was a mesophilic *Rhodopseudomonas* sp. (Uffen, 1976). Since then, other anaerobic CO-oxidizing hydrogenogenic prokaryotes have been described, isolated from a wide range of environments around the world, and spread over different phylogenetic clades (Novikov et al., 2011; Sokolova et al., 2009; Techtmann et al., 2009). The first thermophilic carboxydotroph described was *Carboxydothermus hydrogenoformans* (Henstra and Stams, 2011; Svetlitchnyi et al., 2001). In this work, we describe a novel anaerobic thermophilic carboxydotrophic hydrogenogenic bacteria, strain E3-O, isolated from anaerobic sludge of a municipal solid waste digester. This species can utilize CO as sole carbon and energy source, forming mainly H_2 and CO_2. Phylogenetic analysis based on 16S rRNA gene sequences indicated affiliation to the genus *Moorella*. Strain E3-O was only related to the recognized species of this genus, with 97% 16S rRNA gene sequence identity, and possesses some unique physiological features. Therefore, the creation of a novel species of *Moorella* is proposed.

5.2 Materials and Methods

5.2.1 Enrichments and Isolation

Strain E3-O^T was isolated from a CO-degrading enrichment culture originating from anaerobic suspended sludge from a municipal solid waste digester (Barcelona, Spain). A phosphate-buffered mineral salt medium (20 mM, pH 7.0) was used for enrichment cultures and isolation of strain E3-O^T. The phosphate-buffered mineral medium contained the following components (per liter): Na_2HPO_4, 1.63 g; NaH_2PO_4, 1.02 g; resazurin, 0.5 g; NH_4Cl, 0.3 g; $CaCl_2.2\,H_2O$, 0.11 g; $MgCl_2 \cdot 6\,H_2O$, 0.10 g; NaCl, 0.3 g; 1 mL of acid and alkaline trace element stock each, and 0.2 mL of vitamin stock. Medium was reduced with 0.8 mM sodium sulfide before inoculation. Trace elements and vitamins were prepared as described previously (Stams et al., 1993). Enrichments were performed using CO as sole carbon and energy source. Enrichment cultures were subsequently transferred (10%, v/v) and supplemented with increasing CO partial pressure;

total gas pressure was kept constant at 1.7 bar, and pCO varied from 0.34 bar (CO/N_2 mixture) to 1.7 bar (100% CO). Bottles were incubated in the dark, at 55 °C and 120 rpm. Enrichment of strain E3-O^T was possible by culture dilution series and increasing CO partial pressure, but isolation was only effective after culture autoclaving (2 × 20 min at 121 °C). Purity of the bacterial culture was checked by microscopic examination (Leica DM 2000, Germany).

5.2.2 DNA isolation, PCR-DGGE and sequencing

Direct sequencing of the 16S rRNA gene and denaturing gradient gel electrophoresis (DGGE) were also used to check the genetic purity of the bacterial cultures. Total genomic DNA from cultures of strain E3-O^T was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, USA), according to the manufacturer's instructions. 16S rRNA gene was directly amplified from genomic DNA by PCR, using the primer set 027F/1492R (Nübel et al., 1996) and the following PCR program: pre-denaturation, 2 min at 95 °C; 30 cycles of denaturation, 30 s at 95 °C, annealing, 40 s at 52 °C and elongation, 90 s at 72 °C; and post-elongation, 5 min, at 72 °C. The PCR products were purified using the DNA Clean and Concentrator kit (ZYMO Research, USA) and sequenced directly at BaseClear (Leiden, The Netherlands). Partial sequences were assembled using the alignment editor BioEdit v7.0.9 software package (Hall, 1999). Similarity searches for the 16S rRNA gene sequence derived from strain E3-O^T were performed using the NCBI BLAST search program within the GenBank database (Altschul et al., 1990). Alignment of the 16S rRNA sequences was performed by using the FastAligner V1.03 tool of the ARB program package (Ludwig et al., 2004). The neighbor joining method (Saitou and Nei, 1987) was used for the construction of a 16SrRNA gene based phylogenetic tree.

For DGGE analysis, 16S rRNA gene was partially amplified from genomic DNA with primer set U968GC-f/L1401-r (Lane, 1991; Muyzer et al., 1993). The thermocycling program used for PCR-DGGE amplification was: pre-denaturation, 5 min at 95 °C; 35 cycles of denaturation, 30 s at 95 °C, annealing, 40 s at 56 °C and elongation, 90 s at 72 °C; and post-elongation, 5 min at 72 °C. DGGE was performed using a DCode system (Bio-Rad, Hercules, CA, USA). Gels contained 8% (wt/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) and a linear denaturing gradient of 30-60%, with 100% of denaturant corresponding to 7 M urea and 40% (v/v) formamide. Electrophoresis was performed for 16 h at 85 V and 60 °C in a 0.5× Trisacetate-EDTA buffer. DGGE gels were stained with silver nitrate (Sanguinetti et al., 1994).

5.2.3 Physiological characterization

G+C content determination, DNA-DNA hybridization and cellular fatty acids composition were performed by the identification service of the DSMZ - German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Reference strains *Moorella glycerini* JW/AS-Y6 (=DSM 11254^T) and *Moorella humiferrea* 64-FGQ (=DSM 23265^T) were obtained from DSMZ (Braunschweig, Germany). *M. glycerini*, *M. humiferrea* and strain E3-O were grown

with fructose (20 mM) and yeast extract (0.02%) for determination of membrane fatty-acids composition. Utilization of CO by *M. humiferrea* was also tested using phosphate-buffered medium containing yeast extract (0.02%); bottles contained 10% CO in the headspace (diluted with N_2 to a final pressure of 1.7 bar). Utilization of soluble substrates by strain E3-O, *M. glycerini* JW/AS-Y6 and *M. humiferrea* was performed using a bicarbonate-buffered mineral salt medium (Stams et al., 1993) supplemented with 0.01% yeast extract. Sucrose (20 mM) or glycerol (20 mM) were used as carbon source for testing the utilization of different electron acceptors. The optimum growth temperature (from 15 to 80 °C) and pH (from 5 to 8) was tested with sucrose (20 mM). Sensitivity to antibiotics and to oxygen was also tested using sucrose as carbon source. Antibiotics were added from freshly prepared anoxic filter-sterilized solutions to a final concentration of 100 $\mu g\,mL^{-1}$. The effect of oxygen on the growth of strain E3-O was studied by incubating the culture with different concentrations of oxygen in the headspace, from 2 to 21% O_2 (pO_2 = 0.03 to 0.32 bar). All tests were incubated at least 2 weeks, unless stated otherwise. Growth of strain E3-O was monitored by measuring optical absorbance at 600 nm with a spectrophotometer (U-1500 Hitachi, Tokyo, Japan). Cultures were routinely observed using phase contrast microscopy (Leica DM 2000, Germany).

5.2.4 Analytical analysis

Soluble substrates and intermediates (sugars, volatile fatty acids) were measured using a HPLC Thermo Electron equipment with a Shodex SH1821 column. The mobile phase used was sulfuric acid (0.01 N) at a flow rate of 0.6 $mL\,min^{-1}$. Column temperature was set at 60 °C. Ionic species were analyzed by chromatography using a HPLC DIONEX system, equipped with an Ionpac AS22 column and ED40 electrochemical detector. Column temperature and pressure varied between 35 - 40 °C and 130 - 160 bar. Gaseous compounds (CO,CO_2, H_2) were analyzed by gas chromatography using a GC-2014 Shimadzu equipment, appended with a thermal conductivity detector and equipped with two columns: a CP Poraplot Q column, 25 m × 0.53 mm, df 20 µm, using helium as carrier gas at a flow rate of 15 $mL\,min^{-1}$ and, a Molsieve 13 × column, 2 m × 3 mm, using argon as carrier gas at a flow rate of 50 $mL\,min^{-1}$. For CP Poraplot Q column the temperatures of injector, column and detector were 60, 33 and 130 °C and for the Molsieve column the temperatures were 80, 100 and 130 °C, respectively.

5.2.5 Enzymatic test

Carbon monoxide dehydrogenase (CODH) activity was determined at 55 °C by following spectrophotometrically the CO dependent reduction of oxidized methyl viologen (Svetlitchnyi et al., 2001). For this measurement, cell-free extract was obtained from cultures grown with CO as the only electron donor (pCO = 0.425 bar), and using the procedure previously described by Balk et al. (2009).

5.3 Results and Discussion

A CO-oxidizing bacterium was obtained from a CO-degrading culture enriched from anaerobic sludge of a municipal solid waste digester. Isolation of this bacterium was possible after autoclaving the enriched culture two times, for 20 min at 121 °C and sub-culturing it with CO in the gas phase (pCO = 0.43 bar). A DGGE profile of this culture showed the presence of a single band. Microscopic observations showed that vegetative cells of strain E3-O^T were straight rods, 0.6 - 1 µm by 2 - 3 µm, occurring singly or in pairs (Fig. 5.1).

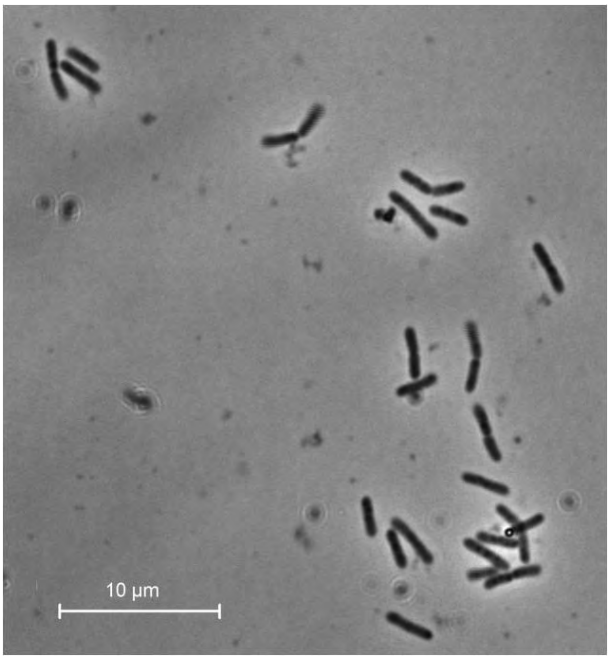


Figure 5.1: Phase-contrast microscopic picture showing the cell morphology of strain E3-O^T. Size bar indicates 10 µm.

Although species from the *Moorella* genus are known to be Gram positive bacteria (Collins et al., 1994), the cells of strain E3-O^T stained Gram variable. This result was always obtained indiscriminately if the cells being grown with CO (gas phase) or with sucrose, and did not change with growth phase of strain E3-O^T. Spores were terminal, round and heat-resistant endospores. Strain E3-O^T could grow between 50 and 70 °C, with an optimum temperature of 65 °C. The optimum pH for growth was 7.5, with a range of 5.7 to 7.8. The doubling time of strain E3-O^T when growing on glucose under optimal conditions was 2.2 ± 0.9 days. Strain E3-O^T could ferment the following substrates (at a concentration of 20 mM): fructose, galactose, glucose, mannose, pyruvate, raffinose, ribose, sucrose and xylose. Slow growth was also observed on arabinose, cellobiose and maltose. Other substrates (at a concentration of 20 mM unless indicated otherwise) were also tested as the sole substrate, but not utilized for growth by strain E3-O^T: acetate, benzoate, butyrate, ethanol, formate, fumarate, glycerol,

lactate, lactose, methanol, propionate, sorbitol, succinate and trehalose, peptone and yeast extract (5 g L^{-1}, each), and H_2/CO_2 (80:20 vol/vol, 1.7 bar). The main product detected from sugar (fructose, glucose, raffinose, sucrose and xylose) and pyruvate fermentation was acetate. Most of the described *Moorella* strains are capable of performing homoacetogenic fermentation of glucose, converting 1 mol of glucose into 3 mol of acetate. Strain E3-O^T converted 1 mol of glucose into 2.16 ± 0.74 mol of acetate. A ratio of 2.31 ± 0.09 mol acetate per mol of glucose has been described for *M. glycerini*, which is the closest relative of strain E3-O^T (Slobodkin et al., 1997). In addition, strain E3-O^T was able to grow on CO as sole carbon and energy source with the production of equimolar amounts of H_2 and CO_2 (Fig. 5.2).

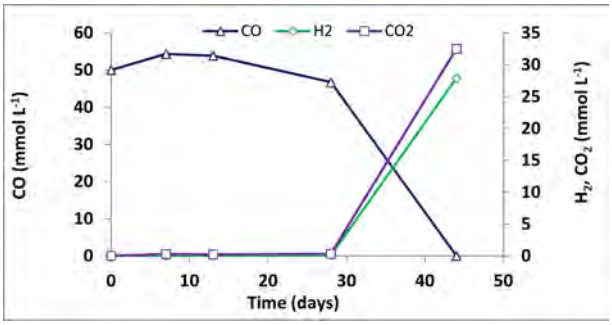


Figure 5.2: Growth of strain E3-O^T in mineral medium under a CO atmosphere (pCO = 1.36 bar). Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using the Henry law. Gas products values are shown as amounts in the gas phase per liter liquid culture.

Moorella species are well known CO-utilizers, but the only hydrogenogenic *Moorella* described thus far is *M. thermoacetica* strain AMP (Balk et al., 2008; Jiang et al., 2009). In agreement with the observed CO-utilization, cell-free extracts from strain E3-O^T were shown to exhibit CODH activity. The maximum CODH activity in cell-free extracts at 55 °C for strain E3-O^T was 15.3 ± 2.6 U mg of $protein^{-1}$. Strain E3-O^T was able to reduce nitrate (20 mM), perchlorate (10 mM) and anthraquinone-2,6-disulfonate (AQDS) (20 mM). The isolate did not reduce sulfate (20 mM), thiosulfate (20 mM) and nitrite (10 mM). Strain E3-O^T can also be distinguished from other *Moorella* species, because all the other described species could use thiosulfate as electron acceptor, and strain E3-O^T could not. Penicillin, ampicillin, chloramphenicol and kanamycin completely inhibited growth 100 µg mL^{-1}. Streptomycin at 100 µg mL^{-1} did not inhibit growth. Strain E3-O^T is an obligate anaerobic microorganism, since there was no growth detected in the presence of oxygen. The cellular fatty acid composition revealed that the most abundant fatty acids of strain E3-O^T were iso-C15:0 (26.18%), iso-C15:0 DMA (15.11 %)and C16:0 (7.11%). Table 5.1 shows a detailed lipid composition of strain E3-O^T. In *M. glycerini*, the predominant fatty acids detected are the same as in strain E3-O^T: iso-C15:0 (37.62%) and iso-C15:0 DMA (18.15%), although the fatty acids composition were less diverse. The fatty

Table 5.1: Cellular fatty acid composition of strain E3-O^T in comparison with phylogenetically closely related species. The strains were grown in bicarbonate-buffered medium supplemented with fructose (20 mM) and yeast extract (0.2 gL^{-1}).

Fatty acids	Strain E3-O^T	*Moorella glycerini* DSM- 11254^T	*Moorella humiferrea* DSM- 23265^T
C14:0	0.71	2.07	0.97
iso-C15:0	**26.18**	**37.62**	**20.58**
anteiso-C15:0	2.23	n.d.	n.d.
C15:0	1.86	n.d.	n.d.
iso-C15:0 DMA	**15.11**	**18.15**	1.60
iso-C16:0	5.39	n.d.	n.d.
C16:0	**7.11**	**10.56**	**21.65**
C16:0 DMA	3.35	2.50	3.29
iso-C17:0	**6.52**	**11.30**	**21.85**
anteiso-C17:0	2.15	n.d.	n.d.
anteiso-C17:0 DMA	2.27	n.d.	n.d.
C17:0 DMA	n.d.	1.15	2.99
C17:0 cyclopropane	n.d.	n.d.	2.21
C18:0 DMA	n.d.	n.d.	1.12
C18:0	1.38	1.79	**13.49**
C18:1 w9c	n.d.	n.d.	0.90
C19:0 cyclo 11-12 DMA	n.d.	1.44	n.d.

n.d.: not detected;

DMA: dimethyl acetal;

Products shown in bold are the predominant fatty acids.

acids C16:0 and iso-C17:0 were substantially more abundant in *M. humiferrea* than in the strain E3-O^T and *M. glycerini*. Besides these differences, iso-C15:0 fatty acid is present in substantial amounts in all the analyzed strains.

The G + C content of the genomic DNA of strain E3-O^T was 54.6 mol%. Phylogenetic analysis of the almost full-length 16S rRNA sequence showed that strain E3-O^T was most closely related to *Moorella glycerini* with 97% 16S rRNA gene identity (Slobodkin et al., 1997), followed by *Moorella humiferrea* with 16S rRNA gene 96% identity (Nepomnyashchaya et al., 2012) (Fig. 5.3). Phenotypically, strain E3-O^T is similar to all other described *Moorella* species, but phylogenetic similarity values between strain E3-O^T and *M. mulderi*, *M. thermoacetica*, *M. thermoautotrophica* and *M. perchloratireducens*, were only among 95 to 93% identity.

Quantitative DNA-DNA hybridization between strain E3-O^T and its closest relative (*M. glycerini*) was performed and the values obtained (in duplicate) were 51.1% - 53.3%, indicating

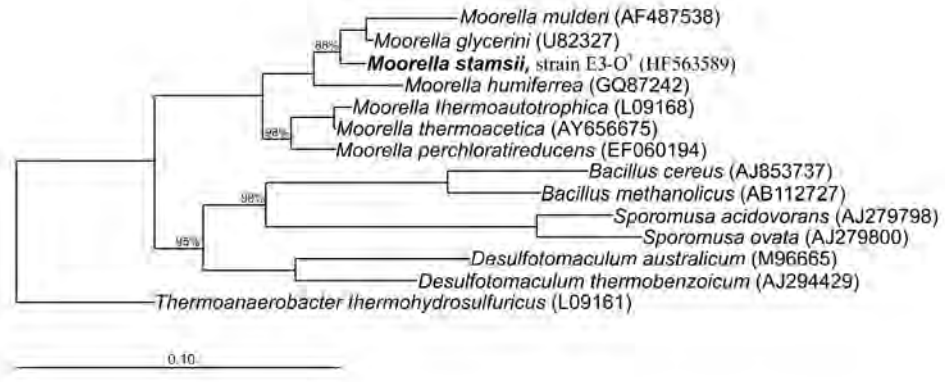


Figure 5.3: Phylogenetic tree of 16S rRNA gene sequences showing the position of strain E3-O^T relative to other species of the genus *Moorella* as well as selected reference sequences of bacteria. The phylogentic tree was calculated using the ARB software package (Saitou and Nei, 1987) and applying the neighbor-joining method with Felsenstein correction. The significance of each branch is indicated at the nodes by bootstrap values (%) based on 1000 replications; only values above 80% are given. GenBank accession numbers of 16S rRNA gene sequences are indicated in parentheses. Bar, 10% sequence divergence.

that strain E3-O^T is a new species of the genus *Moorella*, which was in line with the 97% of 16S rRNA gene identity between *M. glycerini* and strain E3-O^T. Phenotypic characteristics of strain E3-O^T in comparison with the phylogenetically closely related species are presented in Table 5.2. The main differences between strain E3-O^T and the closest related *M. glycerini* were the optimum temperature and pH for growth, but also the conversion of some substrates: strain E3-O^T was able to grow on sucrose but *M. glycerini* not. On the other hand, *M. glycerini* can use glycerol and lactate, but strain E3-O^T did not grow on these two substrates. Furthermore, in contrast to *M. glycerini*, strain E3-O^T can use nitrate and AQDS as electron acceptor. Based on phylogenetic results and physiological properties, it is proposed that strain E3-O^T represents a novel species of genus *Moorella*, *Moorella stamsii* sp. nov.

Table 5.2: Selected morphological and physiological characteristics that differentiates strain E3-O^{T} from its phylogenetic closest relatives. Species: 1 - strain E3-O^{T}; 2 - *Moorella glycerini* DSM 11254^{T} (Slobodkin et al., 1997); 3 - *Moorella humiferrea* DSM 23265^{T} (Nepomnyashchaya et al., 2012).

Characteristics:	1 strain E3-O^{T}	2 *Moorella glycerini* DSM 11254^{T}	3 *Moorella humiferrea* DSM 23265^{T}
Origin	thermophilic anaerobic digester treating organic solid wastes	sediment-water from a hot spring †	terrestrial hydrothermal spring ‡
Optimum temperature ^{o}C	65	58 †	65 ‡
Optimum pH	7.5	6.3-6.5 †	7.0 ‡
Gram reaction	variable	positive †	positive ‡
DNA G+C content (mol%)	54.6	54.5 †	51.0 ‡
Substrate utilization:			
CO	+	+ $^{\#}$	- $^{\diamond}$
Glucose	+	+	-
Mannose	+	+	-
Sucrose	+	-	+
Xylose	+	+	-
Lactate	-	+	+
Glycerol	-	+	+
Electron acceptors:			
AQDS	+	-	+
Nitrate	+	-	+
Thiosulfate	-	+	+

+: utilized; -: not utilized.

† taken from Slobodkin et al. (1997)

‡ taken from Nepomnyashchaya et al. (2012)

$^{\#}$ *M. glycerini* could grow with CO in concentrations up to 50% in the gas phase; no hydrogen production was observed.

$^{\diamond}$ *M. humiferrea* was not able to use CO in concentrations of 10% in the gas phase (after two weeks incubation).

5.4 Description of *Moorella stamsii* sp. nov.

Moorella stamsii (stam'si.i. N.L. masc. gen. n. stamsii, of Stams, named after Alfons J. M. Stams, a Dutch microbiologist, in recognition for his contribution to the advancement in anaerobic microbial physiology).

Cells are straight rods (approx. 0.6-1 μm in diameter and 2-3 μm in length) and show variable response to Gram staining. Usually, cells occur singly or in pairs. Cells produce terminal and round endospores. The most abundant fatty acids are iso-C15:0 (26.18%), iso-C15:0 DMA (15.11%) and C16:0 (7.11%). The G + C content of the DNA of strain E3-O^{T} is 54.6 mol%. The optimum temperature for growth is 65 °C and the optimum pH for growth occurs at pH 7.5. Strain E3-O^{T} is able to grow on (substrates tested in a concentration of 20 mM): fructose, galactose, glucose, mannose, pyruvate, raffinose, ribose, sucrose and xylose. Additionally it can grow on carbon monoxide (100%, pCO=1.7 bar) and produces hydrogen from CO oxidation. Strain E3-O^{T} does not require any growth factors. With acetate, benzoate, butyrate, ethanol, formate, fumarate, glycerol, H_2/CO_2, lactate, lactose, methanol, peptone, propionate, sorbitol, succinate, trehalose and yeast extract no growth was detected. Nitrite, sulfate and thiosulfate could not, but AQDS, nitrate, and perchlorate could act as electron acceptors. E3-O^{T} is an obligate anaerobic bacterium. The type strain is E3-O^{T} (= DSM 26217^{T} = CGMCC 1.5181^{T}), and was isolated in Wageningen, The Netherlands, from a CO-degrading culture enriched from a thermophilic anaerobic suspended sludge of a municipal solid waste digester (Barcelona, Spain).

Chapter 6

Effect of sulfate on CO conversion by a thermophilic enrichment culture

Addition of sulfate to a thermophilic anaerobic syngas degrading stable enrichment led to approximately four times faster CO conversion. Prevalent microorganisms found in cultures with and without sulfate were *Desulfotomaculum* species suggesting that these were responsible for CO conversion, as also described for *D. carboxydivorans*.

Table 6.1: Sulfate-reducing prokaryotes utilizing CO.

Microorganism	Topt. (°C)	Products	Local of isolation source	References
Desulfosporosinus orientis	30-37	H_2S, CO_2	soil	(Stackebrandt et al., 1997)
Desulfovibrio desulfuricans	37	H_2, CO_2, H_2S	oil well corrosion site	(Davidova et al., 1994)
Desulfovibrio vulgaris	37	H_2, CO_2, H_2S	soil	(Lupton et al., 1984)
Desulfotomaculum carboxydivorans	55	H_2, H_2S	anaerobic bioreactor	(Parshina et al., 2005)
Desulfotomaculum kuznetsovii	60	acetate, H_2S	underground thermal mineral water	(Parshina et al., 2005)
Desulfotomaculum nigrificans	55	H_2, H_2S	sewage mud, soil	(Parshina et al., 2010)
Desulfotomaculum thermobenzoicum* ssp. *thermosyntrophicum	55	acetate, H_2S	anaerobic sludge	(Parshina et al., 2010; Plugge et al., 2002)
Archaeoglobus fulgidus	83	acetate, formate, H_2S	submarine hot spring	(Henstra et al., 2007; Stetter, 1988)

6.1 Introduction

Sulfate reducing prokaryotes (SRP) are characterized by the ability of using sulfate as an electron acceptor. These anaerobic microorganisms can be found in many different environments and have a large physiological and phylogenetic diversity. SRP can use a variety of electron donors for growth such as lactate, ethanol, hydrogen and C1-compounds like formate, methanol or carbon monoxide (Muyzer and Stams, 2008; Parshina et al., 2010). Carbon monoxide utilization by thermophilic SRP has been studied as reviewed recently (Oelgeschläger and Rother, 2008; Parshina et al., 2010; Sokolova et al., 2009). Some SRP utilize CO at low concentrations; the sensitivity to elevated levels of CO and consequent growth inhibition if often mentioned (Davidova et al., 1994; Sipma et al., 2006; Sokolova et al., 2009). A number of SRP that utilize CO as sole carbon and energy source (Table 6.1) convert CO to hydrogen and/or acetate, such as *Desulfotomaculum carboxydivorans*, *D. kuznetsovii* and *D. thermobenzoicum* subsp. *thermosyntrophicum* (Oelgeschläger and Rother, 2008; Parshina et al., 2010, 2005; Plugge et al., 2002). *D. carboxydivorans* is the only SRP that can grow with 100% of CO. It produces H_2 both in the presence and in the absence of sulfate. In the obtained syngas-converting culture (T-Syn, chapter 3) the predominant microorganisms present were closely related to *Desulfotomaculum* sp.; we investigated the effect of sulfate on CO degradation by this enriched culture (T-Syn).

6.2 Materials and Methods

6.2.1 Source of thermophilic CO-converting enrichment

The enriched culture degrading syngas, T-Syn, was described in chapter 3. It was obtained by successive transfers of active culture (10% v/v) into fresh medium with the headspace filled with increasing concentrations of CO until reaching 100% of CO, total pressure of 1.7 bar.

6.2.2 Incubation of CO-converting enrichment with sulfate

With the T-Syn enrichment that converted 100% of CO, an experiment to evaluate effect of sulfate on the CO metabolism was performed. The experiments were done in duplicate. Bottles were incubated while stirring (100 rpm) in the dark. A phosphate-buffered (20 mM) mineral salt medium (pH 7.0) was used, as described in chapter 3. A stock solution of sodium sulfate 1 M was prepared and 1 mL of this solution was added to the medium to give a final concentration of 20 mM. All the inoculations and transfers, as well as addition of stock solutions, were performed aseptically using sterile syringes and needles.

6.2.3 Analytical methods

During the experiment, acetate was measured using a Thermo Electron HPLC equipped with a Shodex SH1821 column and a RI detector. The mobile phase used was sulfuric acid (0.01 N) at a flow rate of 0.6 mL min^{-1}. Column temperature was set at 60 °C. Sulfate species were analyzed by using a HPLC Dionex system, equipped with an Ionpac AS22 column and ED40 electrochemical detector. Column temperature and pressure varied between 35-40 °C and 130-160 bar.

Gaseous compounds (CO, CO_2, H_2) were analyzed by gas chromatography on a GC-2014 (Shimadzu) with a thermal conductivity detector. CO_2 was analyzed with a CP Poraplot Q column (25 m length, 0.53 mm internal diameter; film thickness, 20 µm). Helium was used as carrier gas at a flow rate of 15 mL min^{-1}, and the temperatures in the injector, column and detector were 60, 33 and 130 °C. CO and H_2 were analyzed with a Molsieve 13X column (2 m length, 3 mm internal diameter). Argon was used as carrier gas at a flow rate of 50 mL min^{-1}, and temperatures in the injector, column and detector were 80, 100 and 130 °C. Cultures were monitored by microscopic examination during growth with CO (Olympus CX41, Tokyo, Japan).

6.2.4 DNA isolation and PCR-DGGE bacterial profiling

Total genomic DNA from both enrichment cultures: T-Syn - 100% CO, with sulfate and T-Syn - 100% CO, without sulfate, was extracted using a FastDNA SPIN kit for soil (MP Biomedicals, Solon, OH, USA) in accordance with the manufacturer's instructions. Bacterial 16S rRNA genes were amplified by PCR using a Taq DNA polymerase kit (Invitrogen, Carlsbad, CA,

USA); reaction mixtures and PCR programs used were as described elsewhere (Sousa et al., 2009, 2007). Primer sets U968-f/L1401-r and Bact27f/Uni1492r were used for 16S rRNA gene amplification for denaturing gradient gel electrophoresis (DGGE) and for cloning and sequencing purposes, respectively (Lane, 1991; Nübel et al., 1996). A 40 bp GC-clamp was added at the 5' end sequence of the primer U968-f (Muyzer et al., 1993).

DGGE was performed using a DCode system (Bio-Rad, Hercules, CA, USA). Gels contained 8% (wt/vol) polyacrylamide (37.5:1 acrylamide/bis-acrylamide) and a linear denaturing gradient of 30-60%, with 100% of denaturant corresponding to 7 M urea and 40% (vol/vol) formamide. Electrophoresis was performed for 16 h at 85 V and 60 °C in a 0.5× Tris-Acetate-EDTA buffer. DGGE gels were stained with silver nitrate (Sanguinetti et al., 1994).

16S rRNA genes were amplified and PCR products obtained were purified using the PCR Clean Up kit NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). After purification, PCR amplicons were ligated into the pGEM-T vector using the pGEM Easy Vector Systems (Promega, Madison, WI, USA), and introduced into competent *Escherichia coli* 10G (Lucigen Corporation, Middleton, MI, USA), according to the manufacturer's instructions. Positive transformants were selected (by blue/white screening) and grown in appropriate media supplemented with ampicillin. To assign the composition of the predominant community visualized in the DGGE-patterns, nearly full-length bacterial 16S rRNA gene fragments, retrieved from enrichment cultures, were used to construct clone libraries. Clones with the same electrophoretic mobility as that of predominant bands of DGGE-patterns were selected for further sequence analysis.

Sequencing reactions were performed at BaseClear BV (Leiden, The Netherlands) using pGEM-T vector-targeted sequencing primers SP6 and T7 and internal specifically tailored primers, when needed. Partial sequences were assembled by using the Contig Assembly Program (CAP) application included in the BioEdit v7.0.9 software package (Hall, 1999; Huang, 1992). Consensus sequences obtained were checked for potential chimera artifacts using Bellerophon software (Huber et al., 2004). Similarity searches for the 16S rRNA gene sequences derived from the clones were performed using the NCBI BLAST search program within the GenBank database (http://www.ncbi.nlm.nih.gov/blast/) (Altschul et al., 1990).

6.3 Results and Discussion

The enrichment T-Syn (described in chapter 3) was subsequently transferred to new medium with higher CO concentrations reaching 100% of pure CO in the headspace (total pressure of 1.7 bar). During growth and CO conversion at partial pressure of 1.7 bar, also acetate and CO_2 were the main products formed (Figure 6.1). Even though enriched in the absence of sulfate, the predominant microorganisms present in T-Syn culture were closely related to species belonging to the *Desulfotomaculum* genus (see chapter 3). *Desulfotomaculum* species are sulfate

Table 6.2: CO utilization by T-Syn enrichment culture, with and without sulfate.

	T-Syn with sulfate *(≈ 20 mM)*		***T-Syn without sulfate***	
	100% CO (pCO ≈ 1.7 bar)			
Incubation time (days)	30	45	30	45
Substrate utilization (%)	34.1 ± 8.9	74.5 ± 13.4	8.9 ± 4.6	18.3 ± 8.6

reducing bacteria and some members of *Desulfotomaculum* are known for their ability to use and convert CO into hydrogen or acetate (Parshina et al., 2005; Plugge et al., 2002). Thus far, *D. carboxydivorans* is the only *Desulfotomaculum* species that is able to grow with 100% CO in the headspace. The effect of sulfate on CO conversion was tested with the T-Syn culture that grew with 100% of CO in the headspace. With and without sulfate, CO was converted and the main products were acetate and CO_2. However, the CO conversion was approximately 4 times faster in the presence of sulfate (Figure 6.1 and Table 6.2). Approximately 7 mM of sulfate were used by T-Syn, when 75% of CO was already converted (Table 6.3). Under sulfidogenic conditions, from day 0 to day 45, approximately 1.6 bar of CO (corresponding to approximately 73 mmol L^{-1} of CO utilized) was converted into 30 mM of acetate and 40 mmol L^{-1} of CO_2. Acetate was formed from CO according to equation C 6.1 and was further utilized for sulfate reduction. According to equation C 6.2, 7 mM from the produced acetate have been used for sulfate reduction.

$$4\,CO + 2\,H_2O \longrightarrow CH_3COO^- + 2\,CO_2 + H^+ \qquad \text{(C 6.1)}$$

$$CH_3COO^- + 2\,H^+ + SO_4^{2-} \longrightarrow 2\,CO_2 + 2\,H_2O + HS^- \qquad \text{(C 6.2)}$$

Additionally, it was observed higher number of cells in the cultures growing with sulfate; however, the morphology of the dominant microorganisms present in both cultures was the same (Figure 6.2).

Table 6.3: Sulfate utilization by T-Syn enrichment culture.

	T-Syn with sulfate *(≈ 20 mM)*		
	100% CO (pCO ≈ 1.7 bar)		
Incubation time (days)	0	30	45
Sulfate utilization (mM)	0.0	1.5 ± 2.1	6.7 ± 2.2

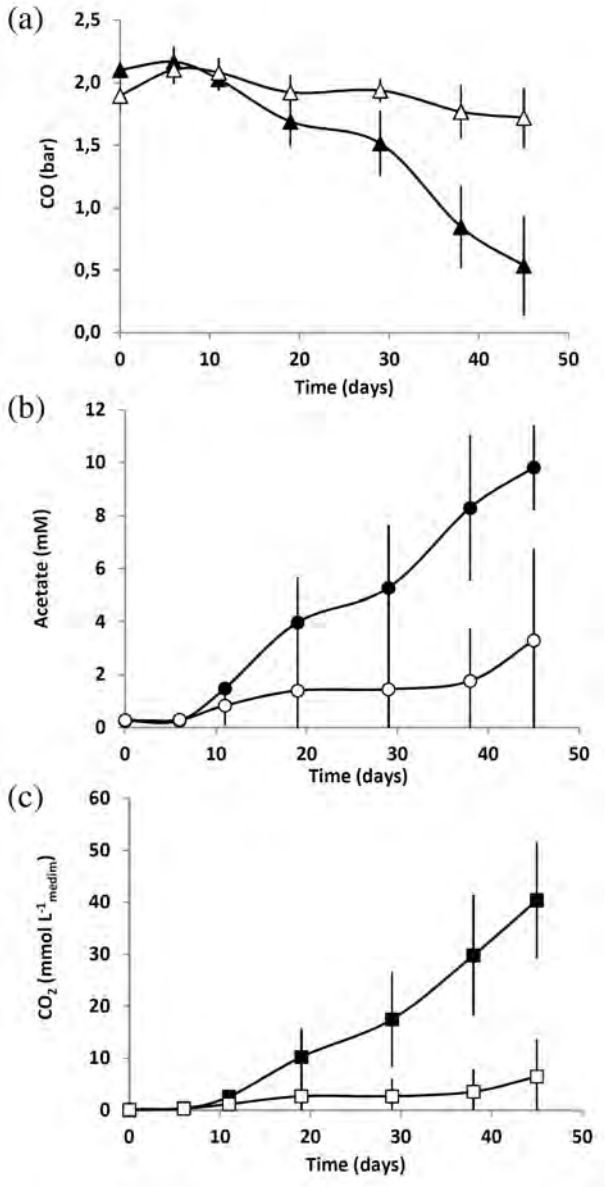


Figure 6.1: CO degradation (a), acetate (b) and CO_2 formation (c) by enriched culture T-Syn with 100% CO, pCO $\approx$ 1.7 bar. Open symbols: medium without sulfate; Filled symbols: medium with sulfate. Total CO_2 was estimated by the sum of the gaseous CO_2 measurement and dissolved CO_2 calculated using the Henry law. The bars indicate maximum and minimum values measured (n=2).

Molecular characterization was also done to investigate the predominant organisms present in the T-Syn cultures growing with 100% of CO) in the present and absence of sulfate. The predominant organisms present in T-Syn with sulfate and in T-Syn without sulfate were the same and were closely related to *Desulfotomaculum* genus (*D. australicum* strain AB33, based on 98% and 97% 16S rRNA gene identity, respectively) (Table 6.4) These selected clones, Cl1-

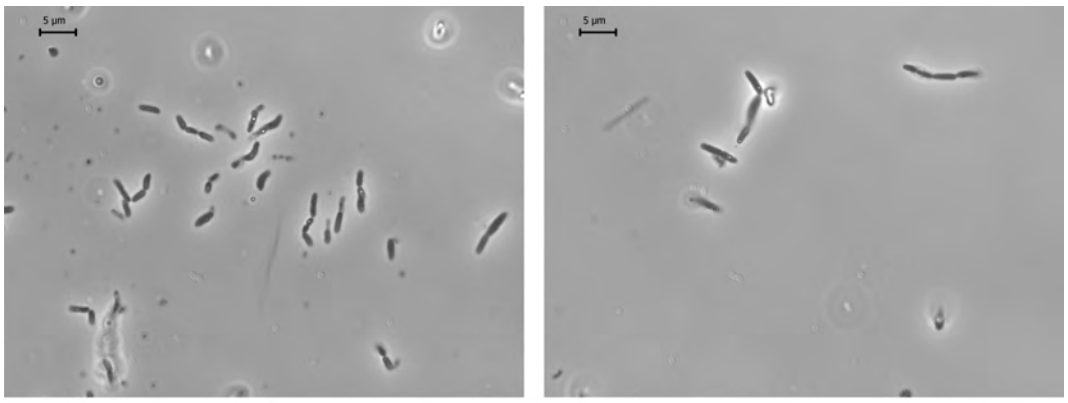


Figure 6.2: Phase contrast microscope pictures of T-Syn culture degrading CO with sulfate (a) and without sulfate (b). Bars: 5 µm.

WS and Cl1-WOS had the same electrophoretic mobility as that of predominant band of in the DGGE-patterns of T-Syn with sulfate and T-Syn without sulfate, respectively. This suggests that the detected *Desulfotomaculum* species present in T-Syn culture are able to convert 100% CO in the presence and in the absence of sulfate. *D. australicum* is a thermophilic SRP that can grow with H_2/CO_2, in the presence of sulfate (Love et al., 1993), but CO utilization by *D. australicum* was never reported. Due to the percentage identity of the 16S rRNA gene sequence of the retrieved clones and *D. australicum* strain AB33 and the *D. australicum* type strain, more research is needed to get insight if the ability to grow with CO in the presence and absence of sulfate is a common property of that species. Genome analysis of *D. carboxydivorans* and *D. nigrificans* suggested that the ability of hydrogenogenic carboxydotrophic growth is associated with the occurrence of a *CooS* gene that has high similarity with the *CooS* gene of *Carboxydothermus hydrogenoformans*, a non-sulfate-reducing thermophile that is also able to grow with 100% CO (Visser et al., 2013 (unpublished); Wu et al., 2005). Although the two phylotypes related to *Desulfotomaculum australicum* AB33 (98% - 97% 16S rRNA gene identity) found in T-Syn enrichments were able to convert CO into acetate, and not into hydrogen under the tested conditions, it would be interesting to explore the possible occurrence of that *CooS* gene in *Desulfotomaculum australicum*.

Table 6.4: Phylogenetic affiliations of cloned 16S rRNA gene sequences corresponding to the predominant clones retrieved from clone library.

Clone ID	Phylum[a]/ Class[a]	Closest relatives	Identity	Acession number
cl1_WS	Firmicutes/	*Desulfotomaculum* sp., clone SYN_1	99%	HF562211
(1585 bp)	Clostridia	*Desulfotomaculum australicum* strain AB33	98%	NR037008
cl1_WOS	Firmicutes/	*Desulfotomaculum* sp., clone SYN_1	99%	HF562211
(1596 bp)	Clostridia	*Desulfotomaculum australicum* strain AB33	97%	NR037008

[a] Classified using the RDP Naïve Bayesian Classifier.

Chapter 7

General conclusions and suggestions for future work

7.1 General conclusions

Biological syngas fermentation processes have been studied over the last few years as a potential alternative route for biofuels production, as reviewed in Chapter 2. In this thesis, microbial physiological aspects of anaerobic syngas and CO conversion were studied. A better insight into the anaerobic conversion of syngas and CO by mixed cultures was obtained and some novel carboxydotrophic organisms were isolated and described. Further investigation on syngas fermentation is needed to open new opportunities for the biological processes as an alternative for the production of biofuels (ethanol, butanol, methane, etc.) or other valuable chemicals (2,3-butanediol, butyric acid, etc.) via the syngas route. The main conclusions of the research are:

i Efficient syngas and CO conversion to acetate or hydrogen is possible under thermophilic conditions by using the same inoculum and applying different startup strategies. The different substrates used (syngas or pure CO as the only carbon and energy source) and the increased CO concentrations applied during the initial transfers of each enrichment series (varying the H_2/CO ratios in syngas mixture), determined the further evolution of the cultures, affecting both the products profile obtained and the microbial composition developed, as highlighted in Chapter 3. Syngas-enriched cultures produced mainly acetate, while CO-enriched cultures mainly formed hydrogen. However, all the obtained cultures could convert syngas and/or CO in concentrations of CO up to 0.88 bar (50% of CO, total pressure of 1.7 bar). Molecular screening of samples from the different enrichments has revealed the predominance of *Peptococcaceae* and *Thermoanaerobacteraceae* members, within the bacterial communities, indicating that they play an important role in syngas and CO conversion. Varying the initial substrate used and also the H_2/CO ratios applied to anaerobic mixed cultures, as startup strategies, could direct the syngas/CO enrichments to the production of a specific product of interest, such as H_2, acetate, methane, etc.

ii From the cultures enriched with syngas or CO two new bacterial species, *Thermoanaerobacter carboxyditolerans* (Chapter 5) and *Moorella stamsii* (Chapter 4), were isolated. *Thermoanaerobacter carboxyditolerans* is a CO-tolerant bacterium that cannot convert CO or syngas, but it is able to grow with sugars at high CO concentrations (100% of CO in the headspace, total pressure 1.7 bar). It was isolated with pyruvate from a syngas-degrading culture. Microorganisms closely related to *Thermoanaerobacter carboxiditolerans* are also tolerant to high concentrations of CO as presented in Chapter 5. *Moorella stamsii* is a CO-converting hydrogenogenic bacterium, isolated from a CO thermophilic enriched culture. Some members of *Moorella* genus are able to use CO as well, converting it into acetate, such as *Moorella thermoacetica* and *Moorella thermoautotrophica*.

iii CO conversion by an enriched culture (T-Syn) was studied in the presence and in the absence

of sulfate, as shown in Chapter 6. In the case of T-Syn culture, where *Desulfotomaculum*-like organisms are predominant, adding sulfate to the medium is a possible way of accelerating CO conversion, since it was observed that CO utilization is faster in the presence of sulfate. Although the main objective of this work was not the study of the biological reduction of sulfate and the use of CO for that purpose, the obtained results gave insight in the potential role of sulfate reducing bacteria (SRB) in biodesulfurization process, using CO as electron donor, and the growth of *Desulfotomaculum*-like organisms with pure CO in the presence and in the absence of sulfate. Furthermore, the results obtained in Chapter 6 emphasize the possible isolation of a new thermophilic carboxydotrophic *Desulfotomaculum* organism from this syngas enriched culture. The SRB *D. carboxydivorans* is the only member of *Desulfotomaculum* genus known to growth with CO as sole carbon and energy source (100% CO in the headspace).

7.2 Suggestions for future work

The work described in this thesis gives insight into the microbiology of thermophilic syngas conversion by anaerobic mixed microbial cultures and by the pure cultures obtained. Most studies describe CO conversion by pure cultures. More effort should be put into screening of microbial mixed cultures able to degrade syngas or CO at thermophilic conditions, since the co-existence of different microbes may lead to divergence of CO conversion through a complex net of reactions or preferential pathways.

Thermophiles are microorganisms that characteristically grow at high temperatures, above 50 °C. Working with thermophiles to produce biofuels and organic solvents from syngas is advantageous over the use of mesophiles. One of the main reasons for that is related to the syngas temperature at the exit of the gasification process, that is in a range of 45 to 55 °C and so, less cooling of the syngas is required before it is introduced into the bioreactor. Additionally, higher temperatures contribute to higher conversion rates and a better separation of the product by distillation (e.g. for ethanol production). Some studies were already performed comparing the syngas and/or CO fermentation process under different conditions. In particular here we present two examples: several anaerobic mixed cultures were tested for their potential to degrade CO at mesophilic (30 °C) and thermophilic (55 °C) conditions by Sipma and co-workers (2003) and significantly higher rates of CO conversion were found under thermophilic conditions; Guiot et al. (2011) also found that the CO conversion to CH_4 was considerably faster under thermophilic conditions as compared to mesophilic operation.

However, there are some disadvantages to the thermophilic processes, namely the lower solubility of gases (CO and H_2) and the low biomass achieved by thermophiles when compared with mesophiles. Despite these facts, the benefit in product recovery improves the overall cost effectiveness of the process. In general, thermophiles have also been considered an important

topic for research because of their biotechnological potential (p.e. due to their thermostable enzymes), and the importance of understanding how life can thrive under extreme conditions and their evolutionary significance. However, the large scale commercial cultivation of thermophiles is still a big challenge.

Additionally, it is of utmost importance to keep making efforts on the isolation and characterization of new thermophilic carboxydotrophic microorganisms. A better understanding of the physiology and biochemical mechanisms of syngas and CO conversion could be achieved by assessing genomic and proteomic information of new and already described thermophilic carboxydotrophic microorganisms.

The CO tolerance tests described in this work showed interesting results for members of *Thermoanaerobacter* genus. Future work on CO tolerance of other groups of interest, both from bacterial and archaeal domains, will give more insights in the mechanisms that microorganisms employ to deal with a high CO concentration. This fact is very important since CO is one of the predominant components in syngas and is also an abundant atmospheric pollutant that is generated by several natural and industrial processes.

Although the main components of syngas are CO, CO_2 and H_2, there are other trace compounds, such as H_2S, NH_3, HCN, NOx, SO_2, hydrocarbons and also tars and ashes, which can interfere with biological processes during syngas fermentation. Little information is available that would allow to infer the effect of impurities on the biological fermentation of syngas. Thus, it is important to assess the influence of impurities present in syngas on the mixed and pure carboxydotrophic cultures.

Syngas can also be fermented to methane in anaerobic processes, although this process is not well studied, yet. Syngas fermentation to methane is not described here, but it represents several interesting aspects, namely upgrading of biogas to biomethane and injection in the natural gas grid. This can make this energy source available for different uses, including transportation. Furthermore, biogas is denser and less toxic than syngas which makes its transport easier, either in pipelines or in tank trucks. Research efforts should also be directed to explore the potential of biogas production from syngas.

7.3 Scientific Output

The overall work presented in this PhD thesis gave origin to the following publications:

Papers in journals with peer review

Alves JI, van Gelder AH, Alves MM, Sousa DZ and Plugge CM. *Moorella stamsii* sp. nov., a new anaerobic thermophilic hydrogenogenic carboxydotroph isolated from digester sludge. June 2013 Int J Syst Evol Microbiol [Epub ahead of print]

Alves JI, Stams AJM, Plugge CM, Alves MM and Sousa DZ. Enrichment of anaerobic syngas converting bacteria from bioreactor sludge. (submitted in FEMS Microbiology Ecology)

Papers in preparation for submission to peer reviewed journals

Alves JI, Stams AJM, Alves MM, Plugge CM and Sousa DZ. *Thermoanaerobacter carboxyditolerans* sp. nov. and comparative analysis of carbon monoxide tolerance by *Thermoanaerobacter* species. (in preparation for submission in Systematic and Applied Microbiology journal)

Publications in conference proceedings

Alves JI, Visser M, Stams AJM, Plugge CM, Alves MM and Sousa DZ. Enrichment and microbial characterization of syngas converting anaerobic cultures (oral communication). Proceeding of the 13th World Congress on Anaerobic Digestion, Santiago de Compostela, Spain, 25th - 28th June, 2013.

Abstracts and posters in conferences

Alves JI, Alves MM, Stams AJM, Plugge CM and Sousa DZ. Isolation and characterization of two novel thermophilic anaerobic bacteria from syngas- and carbon monoxide-degrading cultures, Gordon Research Conference - Molecular Basis of Microbial One-Carbon Metabolism, Lewiston, Maine, USA, 5th - 10th August, 2012.

Alves JI, Pereira FR, Sousa DZ and Alves MM. Mixed culture biotechnology for syngas conversion, 34th Symposium on Biotechnology for Fuels and Chemicals, New Orleans, USA, 30th April - 3rd May, 2012.

Alves JI, Sousa DZ and Alves MM. Microbial syngas conversion by mesophilic and thermophilic anaerobic mixed-cultures, FEMS 2011 - 4th Congress of European Microbiologists, Geneva, Switzerland, 26th - 30th June, 2011.

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